Scanning electron microscopy and swelling test of shrimp shell chitosan and chitosan–RGD scaffolds

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Abstract. Shrimp shell chitosan and chitosan–RGD scaffold membranes are produced to be biocompatible with tissue engineering. Nonetheless, their architectural properties have not yet been studied. Analyze the architectural properties of chitosan and chitosan–RGD scaffolds. Analyze pore count and size, interpore distance, and porosity (using SEM testing and ImageJ analysis) and water absorption (using a swelling test). The properties of the chitosan and chitosan–RGD scaffolds were as follows, respectively. The pore counts were 225 and 153; pore size, 171.4 μm and 180.2 μm; interpore distance, 105.7 μm and 101.4 μm; porosity, 22% and 10.2%; and water absorption, 9.1 mgH2O/mgScaffold and 19.3 mgH2O/mgScaffold. The shrimp shell chitosan–RGD membrane scaffold was found to have architectural properties that make it more conducive to use in tissue engineering.

1. Introduction
Tissue engineering has been undertaken with the aim of treating large bone defects [1]. Bone grafting is one solution for bone defects, but two disadvantages inherent in its use are the relatively low availability of tissue donors and the morbidity of the donor area. A tissue-engineering technique is currently being developed to achieve better treatment outcomes [2].

Tissue engineering requires three main components—namely, cells, signals, and a matrix (i.e., scaffold). The function of a scaffold is to act as a temporary extracellular matrix (ECM) that facilitates cell adhesion, proliferation, and differentiation in the desired tissue contour [1, 2]. In tissue engineering, an appropriate scaffold is needed on which cells can develop, in accordance with the tissue being regenerated [3]. As such, understanding the architectural properties of a scaffold is of great importance. Various architectural properties of a scaffold—such as pore count, pore size, and interpore distance—all affect cell penetration and proliferation. These architectural properties are also linked to the scaffold water absorption rate, which influences cell activity [4–6]. An ideal scaffold must be biocompatible and biodegradable, and approximate the in vivo natural environment [4–7]. A scaffold may comprise polymers and ceramic. Chitosan is a natural polymer that is widely used as a scaffold material; it can be used alone or in combination with other polymers or ceramics as a material used in bone-tissue regeneration [1–5, 8].

Because it is a biodegradable and biocompatible natural polymer, chitosan is used as a scaffold in many industries, including the pharmaceutical, biochemistry, cosmetics, food, and textile industries [1–5, 8]. Chitosan is obtained through chitin deacetylation. Chitin is found in the exoskeletons of crustaceans such as shrimps and crabs, which are fishery products of high economic value [9]. Chitin made from shrimp waste (e.g., heads, shells, and tails) can account for 70% of the shrimp weight, thus
making the shrimp waste industry a potentially accessible source of raw materials in producing chitin and chitosan [8]. As mentioned, chitosan has been proved to be a good scaffold for tissue engineering [1]; however, the use of chitosan does have some disadvantages, given its lack of a bioactive signal for cell adhesion, development, and differentiation. To counter this disadvantage, a chitosan scaffold is usually used in combination with another scaffold, or with an ECM component, to achieve optimal results. One component of an ECM is arginine–glycine–aspartic acid or Arg–Gly–Asp (RGD), which is a cell-binding peptide. The addition of RGD to a chitosan scaffold can increase cell adhesion, development, and differentiation [10].

Jana shows that chitosan may be produced two and three-dimensionally [6]. BATAN Indonesia has produced shrimp shell chitosan–RGD membranes indicated for the repair of large bone defects in periodontal tissues. Additionally, it stated that on a macaque animal model, chitosan–RGD is biocompatible in vivo; however, the architectural properties of the scaffold membrane have yet to be studied [1].

2. Materials and Methods
The current study features a laboratory experiment comprising two tests—namely, SEM testing and swelling test—to determine the properties of chitosan and chitosan–RGD scaffolds.

This research was conducted in June and July 2016. SEM testing and image analysis using ImageJ software were undertaken at the CMPFA Laboratory of the Metallurgy and Material Department of Universitas Indonesia. The swelling test was undertaken at the Oral Biology Laboratory Faculty of Dentistry, at Universitas Indonesia.

The chitosan and chitosan–RGD scaffold membranes samples used in this study were obtained from BATAN ( Jakarta, Indonesia). These scaffolds are made from shrimp shells with a deacetylation degree (DD) of 95%. The chitosan concentration of both scaffolds was 2%. Using a homogenizer set to a speed of 7,500 rpm, 5 mg/50 ml of RGD was added to the chitosan. The scaffolds were then freeze-dried and frozen at –80 °C.

2.1 SEM Testing
The FEI F-50 ( FEI, Eindhoven, Netherlands) brand of FE-SEM was used to undertake SEM testing, as follows. Both scaffold samples were placed on a carbon tape that was adhered to an aluminum disc. An Au–Pd coating was then applied for 60 s, to make the scaffold sample conductive. The aluminum disc containing the samples were then inserted into the FEI F-50, and the environment inside was made a vacuum. Electrons from the FEG were shot towards the conductive samples, producing an SEM image that could be viewed on a computer. SEM images were obtained in soft-copy form, in .JPG format. Four analyses of these image were undertaken with ImageJ software; these used “analyze particle,” to obtain pore count and porosity; “analyze → measure,” to obtain pore size; and “nearest distance,” to obtain interpore distance.

2.2 Swelling Test
The swelling test used 1 mL PBS 0.1 M (pH 7.4) in one of the wells of a 24-well plate. The scaffold was measured dry, with a scale, to a dry weight of 1.4 mg (Wdry). While using a stopwatch, 1 ml of PBS was put into a well and left there for 1 min; it was then removed afterwards for ±30 s, and the scaffold was drained. The wet scaffold was then weighed to obtain the wet weight (Wwet). Measurements were then calculated, using the following formula.

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

After calculating for each of the chitosan scaffold and chitosan–RGD scaffold, their weights were compared.
3. Results and Discussion

3.1 Results

3.1.1 SEM and ImageJ Images

SEM images at magnifications of 150×, 250×, 500×, 1000×, and 2000× were obtained. The image selected for analysis was at 150× magnification, as it adequately showed each pore on the pore surface. When viewed on the computer, it was obvious that the pores were irregular and scattered equally along the whole chitosan and chitosan–RGD scaffold membrane surface (Figures 1 and 2).

![Figure 1. SEM images of chitosan scaffold (A) and chitosan–RGD scaffold (B), at 150× magnification.](image1)

![Figure 2. ImageJ images of pore outlines on the chitosan scaffold (A) and chitosan–RGD scaffold (B), at 150× magnification.](image2)
Analysis using ImageJ software generated results concerning pore count, pore size, interpore distance, and porosity percentage; these are provided in Table 1. As Table 1 shows, the pore counts of the chitosan and chitosan–RGD scaffolds were 225 and 153 pores, respectively. Ten pores representing the pore size were used to generate mean pore sizes for the chitosan and chitosan–RGD scaffolds, of 171.4 μm and 180.2 μm, respectively. The mean interpore distance was calculated by measuring the distances among the four pores closest to one another; the results for the chitosan and chitosan–RGD scaffolds were 105.7 μm and 101.47 μm, respectively. The porosity of the chitosan scaffold was found to be 22.008%, while that of the chitosan–RGD scaffold was 10.138%.

| Group               | Variable | Pore count (quantity) | Mean pore size (μm) | Mean interpore distance (μm) | Porosity (%) |
|---------------------|----------|-----------------------|---------------------|-----------------------------|--------------|
| Chitosan scaffold   |          | 225                   | 171.4               | 105.7                       | 22           |
| Chitosan–RGD scaffold |        | 153                   | 180.2               | 101.4                       | 10.2         |

### 3.1.2 Swelling Test Results

Water absorption was measured by undertaking a swelling test, by using the aforementioned formula. The results of analyses of the chitosan and chitosan–RGD scaffolds are shown in Table 2. The water absorption levels of the chitosan and chitosan–RGD membranes were found to be 9.1 mgH₂O/mgScaffold and 19.3 mgH₂O/mgScaffold, respectively.

| Group               | Variable | Wdry (mg) | Wwet (mg) | Water absorption (mgH₂O/mgScaffold) |
|---------------------|----------|-----------|-----------|-------------------------------------|
| Chitosan scaffold   |          | 1.4       | 14.1      | 9.1                                 |
| Chitosan–RGD scaffold |        | 1.4       | 28.5      | 19.3                                |

### 3.2 Discussion

The results of analysis using ImageJ software showed that the pore count of the chitosan scaffold was 225, whereas that of the chitosan–RGD scaffold was 153 (Table 1). At time of writing, no study had generated an ideal pore count on a scaffold for use in tissue engineering; however, earlier studies have shown that samples with higher pore counts had the advantage of easier cell penetration [11].

This research also used ImageJ software to analyze pore size. The chitosan scaffold was found to have a mean pore size of 171.4 μm, whereas that of the chitosan–RGD scaffold was 180.2 μm (Table 1). These findings are similar to those of New, who states that a high DD will produce pore sizes exceeding 100 μm [11]. According to Laurencin, to achieve optimal results in tissue engineering, pore size must be within the range of 186–200 μm. In the current study, the chitosan and chitosan–RGD
scaffolds were found to have pore sizes 186 μm; this size is ideal, and it is appropriate for use in fibroblast cell proliferation [13]. ImageJ software was also used to undertake analysis of porosity, which is calculated as a percentage of area. The values for the chitosan and chitosan–RGD scaffolds were found to be 22% and 10.2%, respectively (Table 1). These findings run counter to those of earlier studies, which show an ideal scaffold porosity of 90% [2–6]. The thinness of the chitosan scaffold could be one explanation for its low porosity. Meanwhile, low-porosity chitosan scaffolds tend to create stronger chitosan that can withstand loads larger than those high-porosity scaffolds can withstand [12].

Analysis of interpore distance using ImageJ software used, as discussed, the mean distance among four pores. The mean interpore distance of the chitosan scaffold was 105.7 μm, whereas that of the chitosan–RGD scaffold was 101.4 μm (Table 1). Earlier research states that smaller interpore distances may be conducive to better cell proliferation, compared to larger interpore distances [14].

Swelling tests were performed to determine the properties of the scaffolds in terms of water absorption. The chitosan scaffold had a water absorption rate of 9.1 mgH₂O/mgScaffold, while that of the chitosan–RGD scaffold was 19.3 mgH₂O/mgScaffold (Table 2). Clearly, the chitosan–RGD scaffold had a much higher water absorption rate. High water absorption is analogous to high porosity percentage, high pore count, and high pore size [3, 15], and earlier research found that a higher rate of water absorption on a scaffold can result in higher levels of cell interaction [1, 3, 15].

4. Conclusion

The current study obtained data regarding the architectural properties of chitosan and chitosan–RGD scaffold membranes; these included pore count, pore size, interpore distance, porosity, and water absorption. Chitosan–RGD scaffolds were found to have lower pore counts and smaller interpore distances; they were also found to have better water absorption ability, owing to their large pore size. There were some clear differences between the two scaffolds in terms of pore count, pore size, porosity, and water absorption, but differences in terms of interpore distance were not as clear. Overall, however, from the results of the current study, one can draw conclusions regarding the architectural properties of chitosan and chitosan–RGD scaffold membranes. Chitosan–RGD scaffolds have architectural properties that make them more conducive to use in tissue engineering. This research has one particular limitation. The SEM and swelling tests were performed only once each—owing to a limited quantity of scaffold samples—and so no statistical testing could be conducted. Future research should evaluate other architectural properties of chitosan and chitosan–RGD scaffolds, such as pore shape, branching, and pore continuity; the mechanical properties of chitosan and chitosan–RGD scaffolds from shrimp shells should also be studied.

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