

# Modified silk and chitosan scaffolds with collagen assembly for osteoporosis

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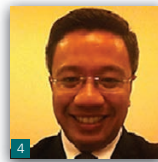
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Currently, many patients suffer from osteoporosis. Osteoporosis is a disease that leads to bone defect. Severe cases of bone defect from osteoporosis need an operation using a performance scaffold for bone tissue engineering. Therefore, to build a performance scaffold for bone defect from osteoporosis is the target of this research. Samples of silk fibroin and chitosan were fabricated into porous scaffolds before modification by coating with collagen self-assembly. The structure and morphology of the samples were characterized and observed by Fourier transform infrared spectroscopy, atomic force microscopy, and scanning electron microscopy. For biological functionality analysis, MC3T3-E1 osteoblasts were cultured on the samples. Afterward, biodegradation, cell proliferation, viability, and mineralization were analyzed. The results demonstrated that collagen organized into a fibril structure covering the pores of the scaffold. The modified scaffolds showed low degradability, high cell proliferation, viability, and mineralization. The results demonstrated that the modified scaffolds with a coating of mimicked collagen self-assembly had good performance and showed promise for bone tissue engineering in osteoporosis.

## 1. Introduction

A report shows that osteoporosis is a major public health problem.<sup>1</sup> About 200 million women around the world have bone loss from osteoporosis, which causes more than 8·9 million fractures annually.<sup>2</sup> The patients who are confronted with osteoporosis must take medications, for example, raloxifene, which is a synthetic estrogen receptor modulator, to preserve the bone; however, its side effects are harmful.<sup>3</sup> Another way to cure the patient is surgery.<sup>4</sup> In some cases the patients need biomaterials for bone substitution.<sup>5</sup> Therefore, to create performance materials for osteoporosis is a challenging issue.

Currently, bone tissue engineering (TE) has been used for bone diseases that need performance material substitution. The aim of bone TE is the repair of damaged tissue and the regeneration of new tissue. The popular approach for bone TE is to seed and culture cells in a porous scaffold before transplanting them into

the target tissue site. The porous scaffold acts as a biodegradable substituted material that has the role of a template for cell adhesion. The cells are then induced to be a new tissue.<sup>6</sup> The scaffold should be biocompatible, be bioresorbable, and have a controllable degradation rate to match the tissue growth. The surface of the scaffold has the proper chemistry for cell attachment, proliferation, and differentiation. The scaffold properties need to be three dimensional (3D) and highly porous with interconnective pores for nutrient and metabolic waste transport.<sup>7</sup>

Silk fibroin (SF) is a protein obtained from the silkworm, *Bombyx mori*. The main amino acids are glycine (43%), alanine (30%), and serine (12%).<sup>8</sup> SF demonstrates excellent properties that include biocompatibility, great mechanical properties, and biodegradability, and SF can be fabricated into various forms.<sup>9</sup> The porous scaffold is suitable for bone TE because the cells

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can proliferate, migrate, and attach to the surface of the interconnecting pores of the scaffold.<sup>10,11</sup>

Chitosan is a semicrystalline polysaccharide. A deacetylated form of chitin is obtained from the cell wall of fungi and the shells of crabs and shrimps and the bony plates of squids and cuttlefish. Pure chitosan has biocompatibility and biodegradability properties and can be prepared in many forms.<sup>12</sup> Because of its unique properties, chitosan has been used as scaffolds for bone TE.<sup>13</sup>

Collagen is a fibril protein composed of a triple helix of the peptide molecule. The main amino acids in the peptide molecule are glycine, alanine, and proline. Predominantly, the fibril structure and amino sequence of arginyglycylaspartic acid in collagen can induce cell adhesion and tissue regeneration.<sup>14,15</sup> The unique biofunctionality of collagen lends itself to be used as material for tissue regeneration. To mimic collagen self-assembly is an attractive technique that can reconstruct the fibril structure of collagen as an extracellular matrix (ECM).<sup>16</sup> Importantly, collagen fibril is the structure that can enhance cell adhesion and proliferation.<sup>17</sup> Therefore, to use the mimicry of collagen self-assembly for scaffold modification was chosen for this research.

Due to the advantages of SF, chitosan, and collagen, they were selected as materials to build a performance porous scaffold for bone TE in this research. SF and chitosan were fabricated into porous scaffolds before modification by coating with mimicked collagen self-assembly that can organize into a fibril structure. The structure, morphology, and biofunctionality of the modified scaffold was considered. The aim of this research is to create a performance scaffold that uses the mimic approach to reconstruct collagen assembly. It is expected that the modified scaffold from this approach holds promise for bone TE in osteoporosis.

## 2. Materials and methods

### 2.1 Materials

#### 2.1.1 Preparation of SF scaffolds

Degummed silk fibrin was extracted by boiling the cocoons for 30 min in 0.02 M sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) to remove sericin, the glue-like protein that holds the fibers together. The degummed SF was dried in a hot oven.<sup>14</sup> A 9.3 M lithium bromide solution was used to dissolve the SF. The solution was then subjected to dialysis to remove the lithium bromide.<sup>15</sup> The SF solution was adjusted to 3% (w/v) and poured into 48 well plates for the forming of 3D SF after the freeze-drying method.<sup>14</sup>

#### 2.1.2 Preparation of type I collagen

The skin of the brown-banded bamboo shark, *Chiloscyllium punctatum*, was used for collagen extraction that followed the report of Kittiphattanabawon *et al.*<sup>16</sup> Briefly, the sharkskin was cut into small sizes, combined with 0.1 M sodium hydroxide (NaOH) to remove the non-collagen proteins. Soaking of the was continued in 0.5 M acetic acid for 48 h. The collagen solution was filtered, and then the final concentration of sodium chloride

(NaCl) was adjusted to 2.6 M and 0.05 M of tris(hydroxymethyl) aminomethane at pH 7.5. The collagen solution was centrifuged using a refrigerated centrifuge machine. Then the collagen pellet was collected and dissolved in a minimum volume of 0.5 M acetic acid. The collagen solution was subjected to dialysis with 0.1 M acetic acid for 12 and 48 h in distilled water. The freeze-drying method was used for removal of the water and kept at  $-20^\circ\text{C}$  until use.

#### 2.1.3 Preparation of chitosan scaffold

Sufficient chitosan powder (Marine Bio Resources Co., Ltd., shrimp chitosan) was dissolved in 0.1 M acetic acid pH 4.5 to a 2% concentration and mixed continuously in a magnetic stirrer for 24 h. The chitosan solution was poured into 48 well plates and then kept at  $-20^\circ\text{C}$  overnight. The freeze-drying method was used to fabricate 3D chitosan scaffolds.<sup>17</sup> After that, they were cut into 10-mm-dia. and 2-mm-thick pieces.

#### 2.1.4 Modification of SF and chitosan scaffolds

This study designed the scaffolds into four groups: (a) non-coated SF scaffolds without collagen, (b) coated SF scaffolds with collagen, (c) non-coated chitosan scaffolds with collagen, and (d) coated chitosan scaffolds with collagen. Used for coating was 0.1 mg/ml collagen solution (Table 1). To coat with the collagen solution, SF and chitosan scaffolds were immersed in the collagen solution for 4 h at  $37^\circ\text{C}$ . Afterward, the immersed scaffolds were soaked in  $1\times$  phosphate buffered saline (PBS) for 30 min to form self-assembly of collagen. These scaffolds were kept at  $-20^\circ\text{C}$  for overnight before freeze-drying.

## 2.2 Methods

### 2.2.1 Self-assembly of type I collagen

To observe self-assembly, 0.1 mg/ml collagen solution was mixed with PBS pH 7 at the final concentration of 0.05 mg/ml. The optical density (OD) at 313 nm was used to identify the form of the collagen fibrils.<sup>18</sup> The OD of the mixed collagen solution was measured every 5 min for 30 min. The OD of each time point was plotted into a kinetic curve to explain the collagen self-assembly.

### 2.2.2 Atomic force microscopy (AFM)

A sample of the collagen solution at a concentration of 0.1 mg/ml was dropped and smeared onto a glass slide. After soaking in  $1\times$  PBS for 30 min, the glass slide was dried at room temperature. Then, the coated glass slide with collagen was observed for self-assembly formation of the collagen by AFM (Nanosurf Easyscan 2 AFM, Switzerland).

Group	Detail
A	SF scaffold
B	Coated SF scaffold with collagen
C	Chitosan scaffold
D	Coated chitosan scaffold with collagen

Table 1. Groups of scaffolds

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### 2.2.3 Fourier transform infrared (FTIR) spectroscopy

The chemical functional group of collagen was obtained using an FTIR spectrometer (Equinox 55, Bruker, Ettlingen, Germany). The internal reflection crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45° angle of incidence of the infrared (IR) beam. The spectra were acquired at a resolution of 4 cm<sup>-1</sup>. The spectral data analysis used the Opus 3.0 data collection software program (Bruker, Ettlingen, Germany). To characterize the chemical function groups of collagen self-assembly, the mixed collagen with PBS as in the previous experiment was freeze-dried before preparation into potassium bromide (KBr) disks and measured by FTIR. Coated SF and chitosan scaffolds were measured by FTIR in attenuated total reflectance mode.

### 2.2.4 Scanning electron microscopy (SEM)

All groups of scaffolds were observed for morphology, surface, and pore size by a scanning electron microscope (Quanta 400, FEI, Brno, Czech Republic). The samples were precoated with gold using a gold sputter coater machine (SPI Supplies, division of Structure Probe Inc., Westchester, PA, USA).

### 2.2.5 Degradation

Lysozyme powder was mixed with PBS into solution at 4 mg/ml (pH = 7.4) before incubation at 37°C.<sup>19</sup> The scaffolds were immersed in the solution. The scaffolds were then removed from the solution, rinsed, and freeze-dried. The freeze-dried scaffolds were weighed at different time points: 1, 2, and 4 weeks. Afterward, the percentage of weight loss was calculated.

## 2.3 Cell culture

MC3T3-E1 osteoblast cells were seeded in each scaffold with 1 × 10<sup>5</sup> cells and maintained in an  $\alpha$ -minimum essential medium (Gibco, Invitrogen, Carlsbad, CA, USA) with the addition of 1% penicillin/streptomycin, 0.1% fungizone, and 10% fetal bovine serum at 37°C in a humidified 5% carbon dioxide (CO<sub>2</sub>) and 95% air incubator.<sup>20</sup> The medium was changed every 3–4 d. An osteogenic (OS) medium (20 mM  $\beta$ -glycerophosphate, 50  $\mu$ M ascorbic acid, and 100 nM dexamethasone; Sigma-Aldrich) was used for osteoblast differentiation of the MC3T3-E1 osteoblast cells.<sup>21</sup>

### 2.3.1 Cell proliferation

The measurement of cell proliferation was performed on days 3, 5, and 7.<sup>22</sup> Following the manufacturer's protocol, the scaffold was washed two times with 1× PBS, and fresh media of 100  $\mu$ l and 10  $\mu$ l of 12 mM 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyl tetrazolium bromide (MTT; 5 mg/ml) were added into the cells and scaffolds, respectively. Afterward, the cells and scaffolds were incubated for 2 h at 37°C. Then, 50  $\mu$ l of dimethyl sulfoxide was added to each cell and scaffold and incubated for 10 min. The solutions were moved to 96 well plates and measurements continued by monitoring the light absorbance at 540 nm.

### 2.3.2 Cell viability

On day 3, the MC3T3-E1 osteoblast cells in the scaffolds were stained with fluorescein diacetate (FDA). FDA attached to the

ECM and cellular clusters. FDA was dissolved in acetone at a concentration of 5 mg/ml. The medium was removed and replaced with 1 ml of fresh medium, and then 5  $\mu$ l of FDA was added. The scaffolds were kept away from light for 5 min. The scaffolds were washed twice with 1× PBS and moved to a glass slide, and the cell morphology was observed by a fluorescence microscope.<sup>23</sup>

### 2.3.3 Alizarin red staining

The calcium synthesis of the MC3T3-E1 osteoblast cells was inspected by alizarin red staining. On day 7, the scaffolds were washed with 1× PBS and the cells were fixed with 4% formaldehyde before the addition of 1 ml of alizarin red solution (2 g in 100 ml of distilled water to adjust the pH to 4.1–4.3) for 20 min at room temperature in the dark.<sup>24</sup> The alizarin red was removed carefully from 48 well plates, and the scaffolds were washed with distilled water until the red color disappeared. Afterward, the scaffolds were observed by light microscope.

### 2.3.4 Statistical analysis

All data were shown as mean  $\pm$  standard deviation. The samples were measured and statistically compared by one-way analysis of variance and Tukey's honest significant difference test (SPSS 16.0 software package).  $P < 0.05$  was accepted as statistically significant.

## 3. Results and discussion

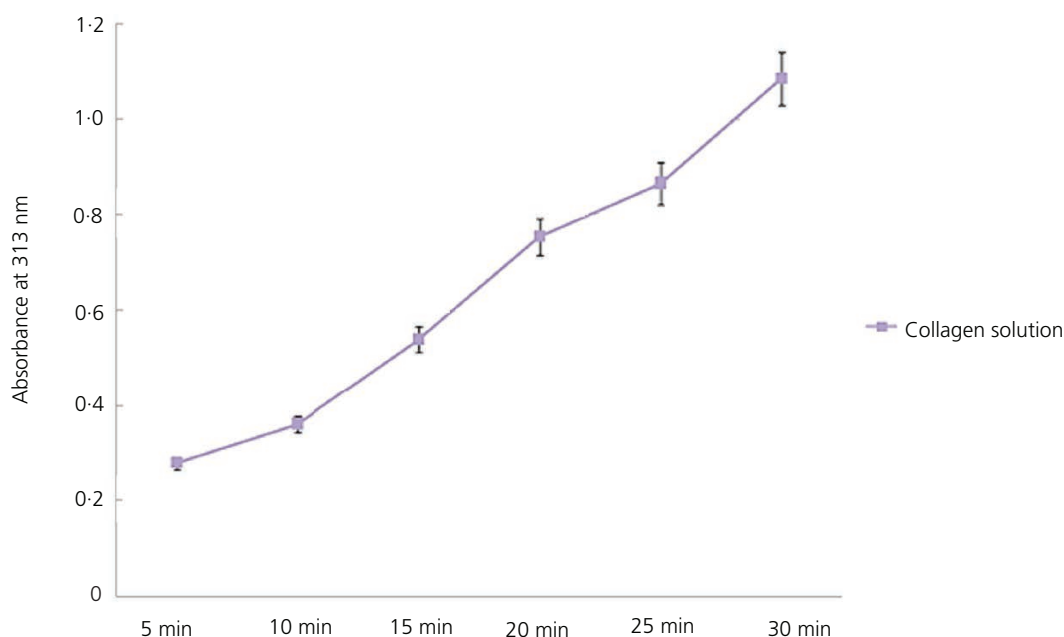
### 3.1 Self-assembly of collagen fibril

Before coating the scaffolds, the self-assembly of collagen was monitored by measuring the absorbance at a wavelength of 313 nm at each time point. Then, the absorbances were plotted into a kinetic curve (Figure 1). The curve represented the self-assembly of collagen fibrils.<sup>25</sup> The collagen self-assembly in solution was monitored for 30 min, which corresponded to the coating time of collagen on the scaffolds. The absorbance value increased with time (Figure 1). During the time from 5 to 30 min, the group absorbance was higher at each time point, which meant collagen fibrils were forming in the solution. In this study, 0.1 mg/ml type I collagen solution (0.1 M acetic acid, pH 2.88) was mixed with PBS at a ratio of 1:1 for neutralization. Under these conditions, collagen molecules organized and aggregated into the fibril structure.<sup>26</sup> Notably, this indicated that the collagen organized into the fibril structure during the time of coating.

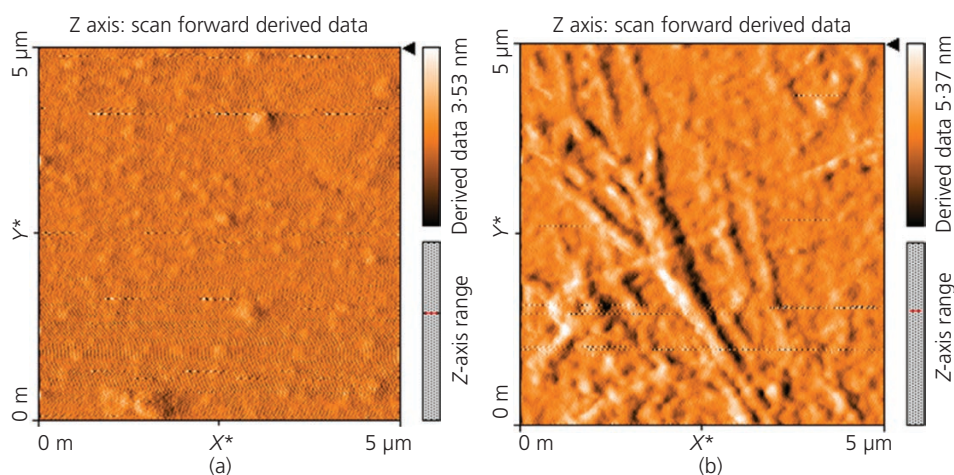
### 3.2 Collagen self-assembly by AFM

The neutralized collagen solution was dripped and dried on a glass slide to observe the structure formation by AFM of the collagen fibrils in the coating. The collagen fibrils organized themselves into small branches (Figure 2). Interestingly, this indicated that the neutralized collagen solution had produced coating that could mimic the fibril structure as an ECM. Notably, the mimicked collagen fibril could induce cell adhesion and proliferation.<sup>27</sup> Nevertheless, to confirm the fibril structure of collagen, the neutralized collagen solution was freeze-dried before characterization by FTIR in the next section.

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**Figure 1.** Kinetic curve of self-assembly of collagen measured by absorbance at 313 nm against time (min)



**Figure 2.** Self-assembly of collagen into fibrils observed by AFM: (a) glass slide with non-treated collagen coatings and (b) collagen bundles on the glass slide immersed in PBS

### 3.3 FTIR analysis

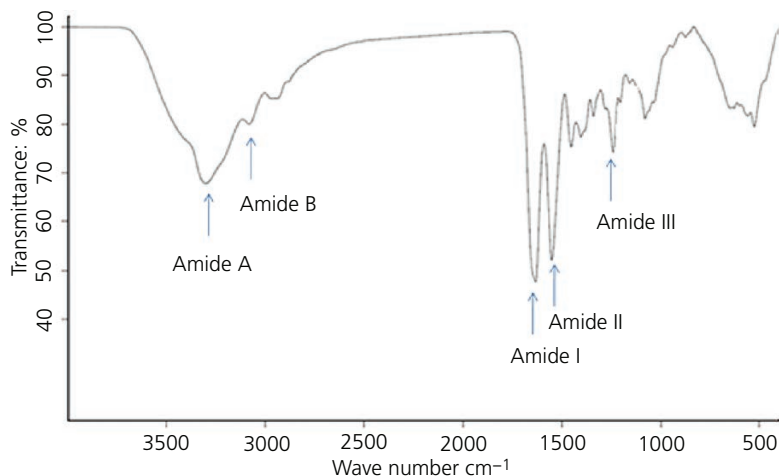
The freeze-dried neutralized collagen solution was characterized by FTIR to demonstrate the fibril structure of the collagen coating (Figure 3). Principally, the FTIR technique detected the vibration characteristics of the chemical functional groups of collagen. A specific wave number ( $\text{cm}^{-1}$ ) range of IR radiation was absorbed by the chemical functional group.<sup>28</sup> The amide A band of collagen was found at  $3292 \text{ cm}^{-1}$ ; this was the general band associated with the N-H stretching vibration and indicated the existence of hydrogen bonds. When the NH group of peptides formed the

hydrogen bond, the absorbance shifted to a lower wave number. The amide B band was observed at  $2921\text{--}2925 \text{ cm}^{-1}$ . The amide I band of collagen was found at  $1631 \text{ cm}^{-1}$ . This band was due to C=O stretching vibration. Importantly, the FTIR results indicated that the collagen could organize into fibril structures.<sup>22</sup> Therefore, these results confirmed the previous explanation that collagen could form into a fibril structure in the coating.

The samples were measured by FTIR to characterize the existence of collagen on SF and chitosan scaffolds. The absorption points



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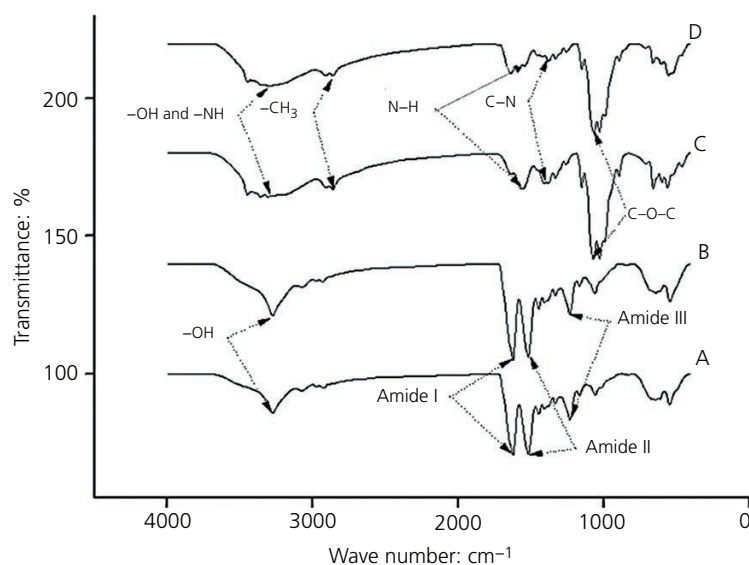
**Figure 3.** FTIR spectrum of collagen fibrils after freeze-drying

were around  $1622$  and  $1517\text{ cm}^{-1}$ , which indicated amide I and II (N–H) (Figure 4). The amide III (C–N) of SF was absorbed at  $1234\text{ cm}^{-1}$ .<sup>29</sup> For the coated SF scaffold, the collagen fibrils were distributed on the silk scaffold. This affected the merged peak of SF and collagen.<sup>30</sup> In the case of the chitosan scaffolds with and without coating, the peaks of the –OH and –NH groups appeared at around  $3360\text{ cm}^{-1}$ . Notably, the N–H group bending of chitosan without coating was identified with the spectra absorption band at  $1554\text{ cm}^{-1}$ , which was different from the spectra absorption bands of coated chitosan scaffold with collagen at  $1637$ ,  $1546$ , and  $1257\text{ cm}^{-1}$ , which were characteristic of the amide I (C=O), II (N–H), and III (C–N) bands, respectively. Those three peaks

showed the characteristics of collagen. The results from the FTIR characterization demonstrated that collagen existed on the scaffolds particularly in chitosan. However, to confirm the existence of collagen on the scaffolds, the samples were observed by SEM analysis.

### 3.4 SEM analysis

After clarification that the collagen could arrange into a fibril structure, the coating solution was used for SF and chitosan scaffolds. For coating, SF and chitosan scaffolds were immersed in a collagen solution at pH 3 before soaking in PBS. Then, those scaffolds were freeze-dried before observation of the morphology



**Figure 4.** FTIR spectrum of coated scaffolds with collagen fibrils: SF scaffold without coating (A), SF scaffold with collagen coating (B), chitosan fibroin scaffold without coating (C), and chitosan scaffold with collagen coating (D)

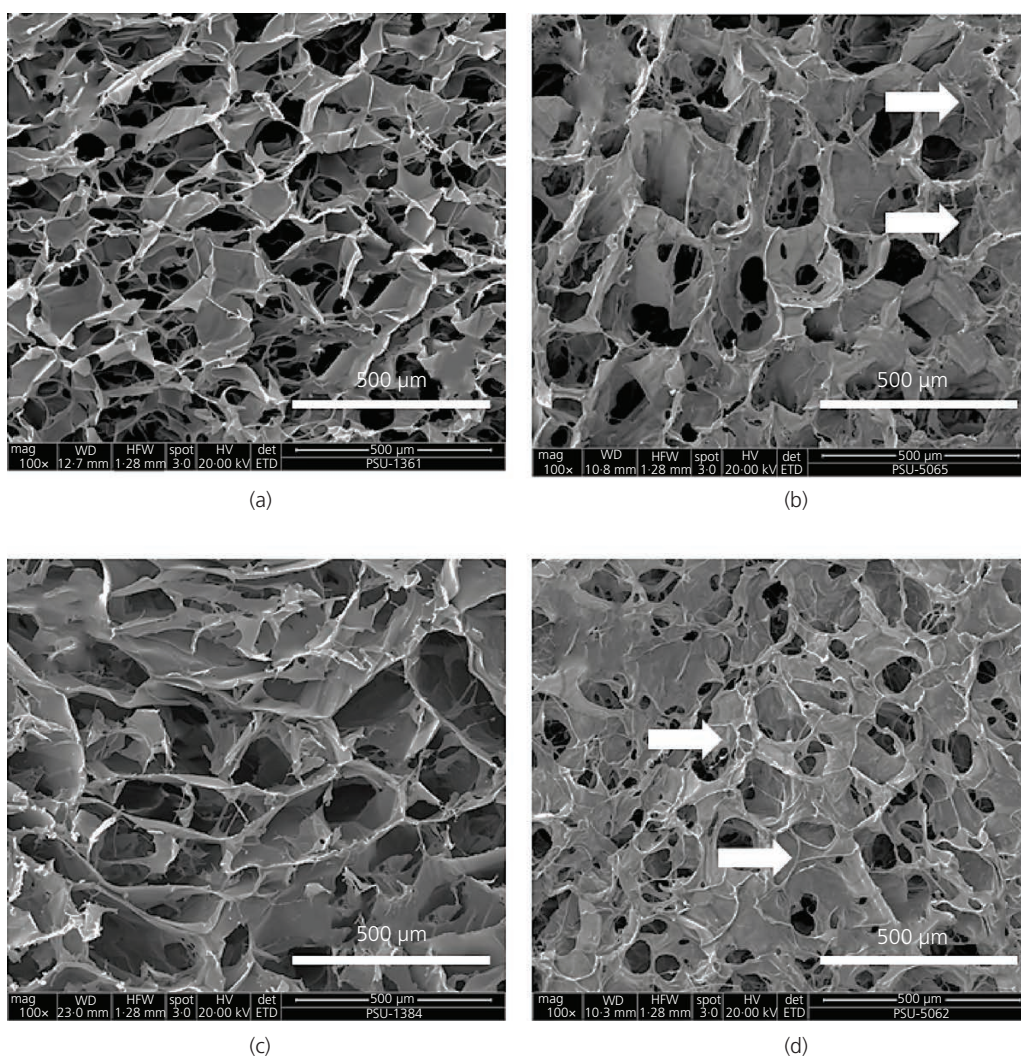
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by SEM. Interestingly, the morphology of the scaffolds showed that the coated scaffolds of SF and chitosan had deposited a fibril network structure of collagen inside the pores (Figure 5). Therefore, the results from the SEM indicate that collagen could form a fibril network structure as a mimicked ECM that deposited inside the pores of the scaffolds. Importantly, the mimicked ECM might induce cell adhesion and proliferation as according to a previous report.<sup>31</sup> Besides the suitable structure for cell adhesion and proliferation, it is important to determine the biodegradation of scaffolds in TE. Biodegradation and cell experiments were undertaken to vindicate those issues.

### 3.5 Analysis of scaffold degradation

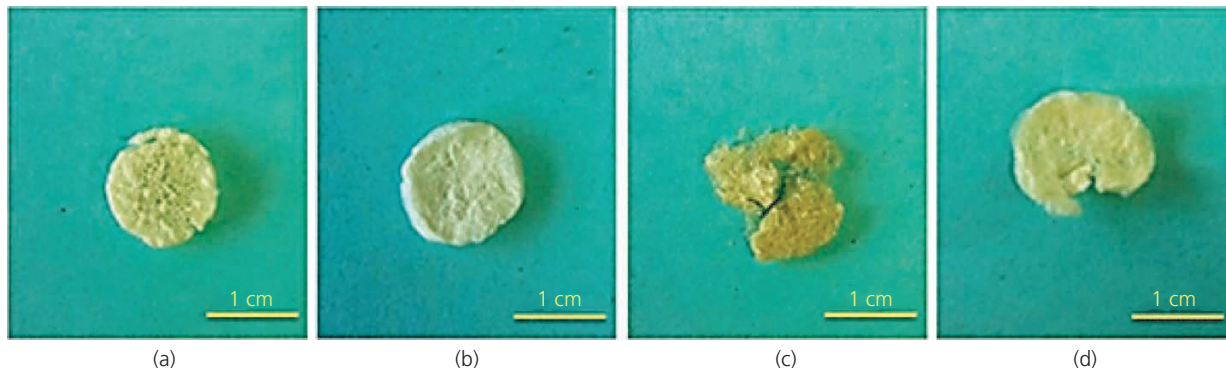
All scaffolds were tested with lysozyme to analyze scaffold degradation as in Section 2.2.5. The scaffolds in all groups showed a changed shape. The silk scaffold revealed the surface and margin

areas that were digested with lysozyme (Figure 6(a)). The surface area of the silk scaffold coated collagen group collapsed but maintained a good shape (Figure 6(b)) when compared with the other groups. The chitosan scaffold was broken after digestion and the surface and margin areas were digested (Figure 6(c)). The coated chitosan scaffold with collagen showed the most digestion in the marginal zone, and the surface area collapsed after digestion (Figure 6(d)). Both silk and chitosan scaffolds coated with collagen showed slow degradation compared to the non-coated scaffolds. The triple-helix structure of collagen coated on the scaffold surface was the cause of the difficult degradation. The SF scaffold coated with collagen showed the least amount of degradation. These results illustrated the same explanation as previously reported that the molecules of the enzyme had less opportunity to contact the scaffold.<sup>32</sup> Furthermore, the literature was reported that the SF could extend biodegradability of the scaffolds.<sup>33</sup>



**Figure 5.** Morphology and surface of scaffold in each group observed by SEM: (a) SF scaffold, (b) collagen-coated SF scaffold, (c) chitosan scaffold, and (d) collagen-coated chitosan scaffold

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**Figure 6.** Scaffolds after degradation with lysozyme at 4 weeks: (a) SF scaffold, (b) collagen-coated SF scaffold, (c) chitosan scaffold, and (d) collagen-coated chitosan

The SF scaffolds with and without modification had more stability from biodegradation than the modified and non-modified chitosan scaffolds. Importantly, the results indicated that collagen could improve biodegradation of scaffolds. Interestingly, SF and chitosan scaffolds tolerated better the enzyme activity after coating (Figure 7). The results of biodegradation indicated that the coated scaffolds with mimicked collagen self-assembly had a suitable performance for bone TE. However, to confirm the performance of those modified scaffolds, experiments to determine cell proliferation, viability, and mineralization were undertaken.

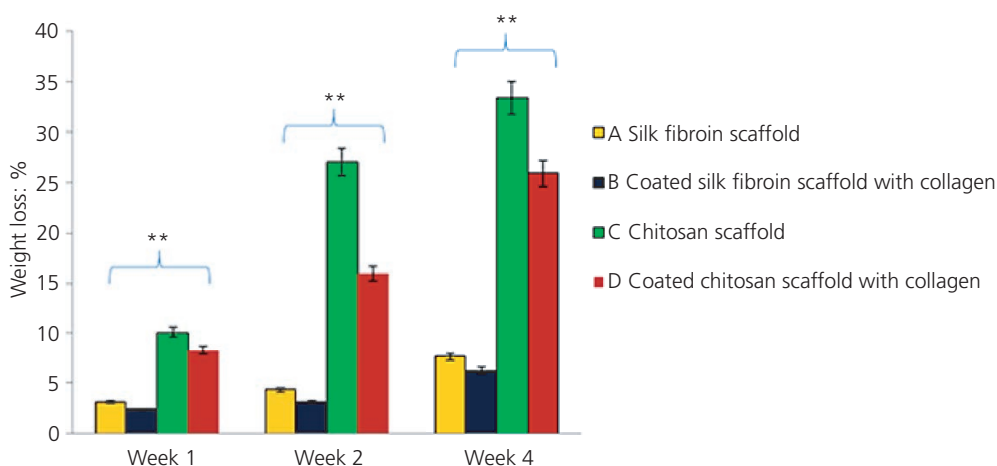
### 3.6 Cell proliferation

Figure 8 shows the MTT assay of the cell proliferation on the scaffolds. The results showed that the OD values increased from days 3 to 5 and then decreased on day 7 of the cell culture. On day 3, the OD value of the SF scaffold was higher than that of the chitosan scaffold. The coated scaffold with mimicked

collagen self-assembly had directly improved cell proliferation. The cell proliferation of the SF and chitosan scaffolds after coating with mimicked collagen self-assembly showed good performance for bone TE. Cell proliferation in all groups of the scaffold increased on day 5, which demonstrated that the cells adhered and proliferated in all groups. Notably, the collagen-coated SF scaffold and the collagen-coated chitosan scaffold showed the highest cell proliferations on day 5. The results indicated that collagen promoted cell proliferation and adhesion. The literature reported that collagen had the important role of inducing cell migration and differentiation.<sup>34</sup>

### 3.7 Fluorescein diacetate

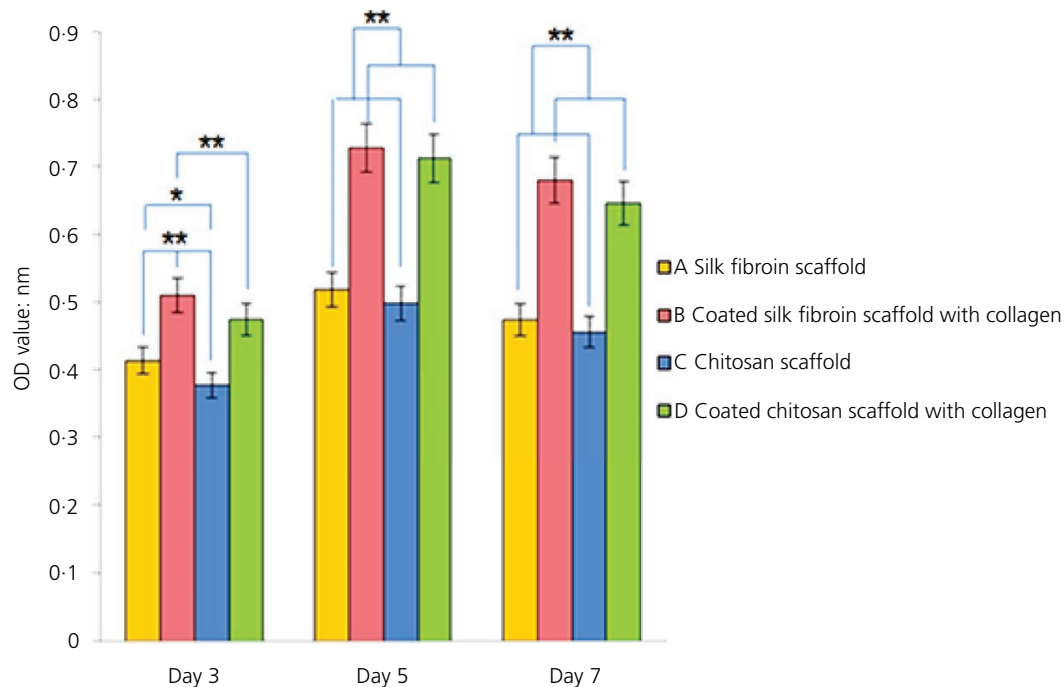
The MC3T3-E1 cells adhered in all groups to the scaffolds (Figure 9). The bright green fluorescence indicated the cell viability and morphology thoroughly on the surface. The coated scaffolds with mimicked collagen self-assembly showed several cells compared to the non-coated scaffolds. The cells arranged



**Figure 7.** Degradation of scaffold after digestion with lysozyme at weeks 1, 2, and 4. The symbol (\*\*,  $P < 0.01$ ) represents significant changes in weight losing of scaffold in each group

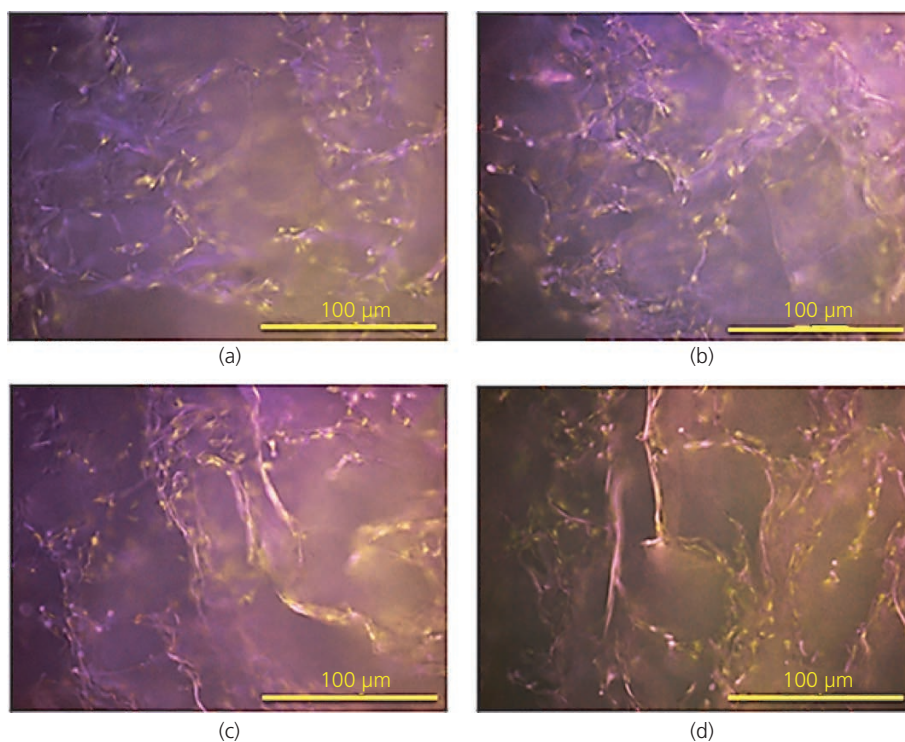


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**Figure 8.** MTT assay of MC3T3-E1 grown on various scaffolds at days 3, 5 and 7. The symbol (\*) represents significant changes in MTT assay

activity of the MC3T3-E1 osteoblasts grown on various scaffold ( $P < 0.05$ ), (\*\*) ( $P < 0.01$ )



**Figure 9.** Fluorescence image showing the viability (bright green) of MC3T3-E1 attached to the scaffolds in all groups: (a) SF scaffold, (b) collagen-coated SF, (c) chitosan scaffold, and (d) collagen-coated chitosan

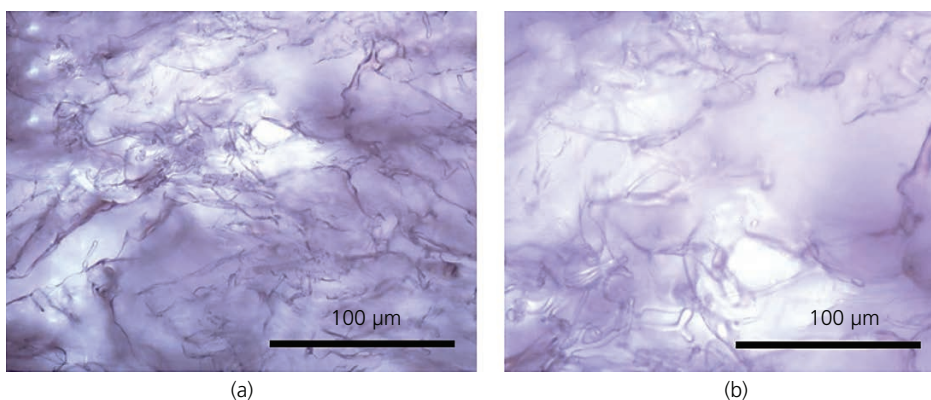


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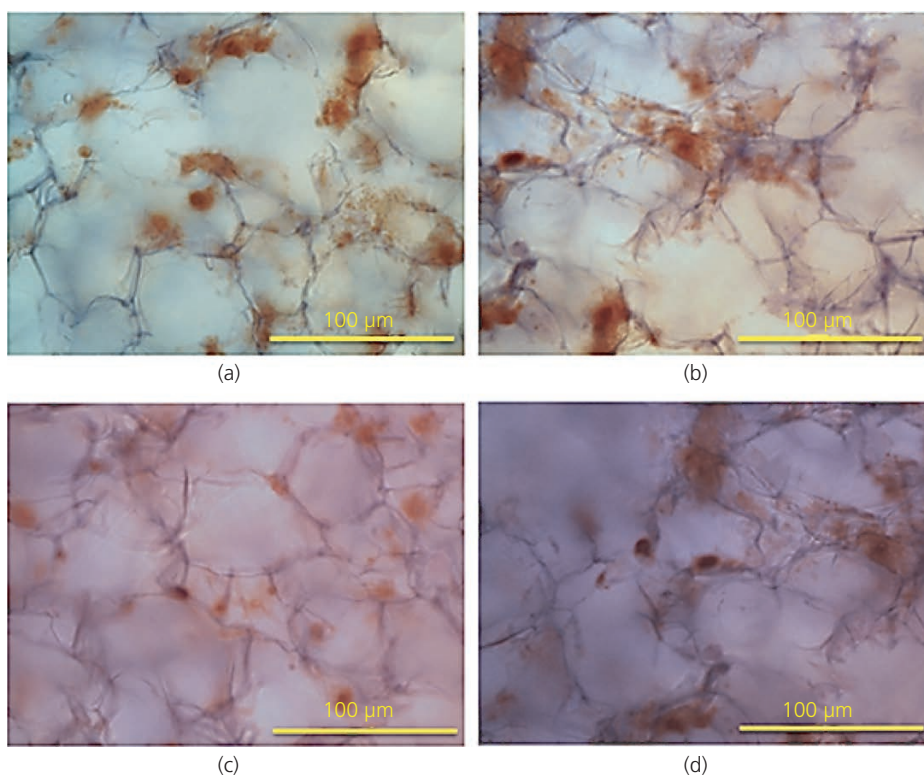
and expanded themselves on the surface of the coated scaffolds. This demonstrated that the coated scaffolds with mimicked collagen self-assembly could enhance cell viability. However, to confirm the performance of scaffolds for bone TE, the presence of calcium in the scaffold was analyzed and observed in the next section.

### 3.8 Alizarin red

To confirm the presence of calcium that was secreted from the MC3T3-E1 cells, the scaffolds were stained with alizarin red. Afterward, the stained scaffolds were observed by microscope. Calcium nodules were found in all groups of scaffolds (Figures 10 and 11). The results showed that the MC3T3-E1 cells could



**Figure 10.** Alizarin red staining of the scaffolds without cell culturing: (a) SF scaffold and (b) collagen-coated SF



**Figure 11.** Alizarin red staining of the scaffolds at day 7 of cell culture under OS media conditions. Red indicates calcium deposits on the scaffold. (a) SF scaffold; (b) collagen-coated SF scaffold; (c) chitosan scaffold; (d) collagen-coated chitosan scaffold

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grow in the scaffolds and secrete calcium onto the scaffolds. The staining with alizarin red indicated a high amount of calcium deposition. The coated scaffolds could induce calcium synthesis from the MC3T3-E1 cells. Notably, in the coated SF, the calcium deposition was more intensive than that in the coated chitosan scaffold. The results demonstrated that the coated scaffolds with mimicked collagen self-assembly had a suitable performance for bone TE, particularly the coated SF scaffold.

#### 4. Conclusion

The use of modified scaffolds by coating with mimicked collagen self-assembly for TE was proposed in this research for osteoporosis treatment in the case of bone defect. The results of this research indicated that collagen organized into assembled fibril structures in the pores of the coated scaffolds. The fibril structures showed performance as an ECM that could induce biological functionalities of coated scaffolds. Predominantly, the coated SF and chitosan scaffolds with collagen self-assembly had good biological functionalities: stability from biodegradation, good cell proliferation, viability, and mineralization. Importantly, it can be deduced that the scaffolds modified by coating with mimicked collagen self-assembly had a suitable performance for bone TE and showed promise for use in osteoporosis treatment.

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