Characterization of Porous Scaffold from Chitosan-Gelatin/Hydroxyapatite for Bone Grafting

Wassanai Wattanutchariya and Whattanapong Changkowchai

Abstract— **Bone tissue engineering is a new treatment technique for bone grafting. This procedure can regenerate damaged bone by implanting scaffolding to provide mechanical support in gap areas. The scaffold acts as a temporary matrix for cell proliferation until new bone tissue is completely regenerated. This research developed bone scaffold using freeze-drying method. A mixture design technique was used to investigate the effect of chitosan, gelatin, and hydroxyapatite on the scaffold properties. The results showed that the degradability and porosity of the scaffold increased with decreasing chitosan-gelatin and hydroxyapatite concentrations, while swelling increased with increasing chitosan-gelatin but decreasing hydroxyapatite concentrations. An optimal condition was obtained from the scaffold with a chitosan-gelatin:hydroxyapatite:1% acetic acid ratio of 2.62:2.17:95.21. The SEM image also showed the scaffold fabricated from this ratio has an open pore structure, which could benefit bone regeneration.**

Index Terms— **Chitosan, Gelatin, Hydroxyapatite, Porous scaffold, Bone grafting**

I. Introduction

BONES are rigid organs that support and protect various organs of the body. Repair techniques for bones with defects or loss due to disease, trauma or tumor resections [1] are the subject of intensive research. Bone grafts – a well-recognized and standard treatment method for reconstructive orthopedic surgery [2] – employ three types of bone or tissue substitution: allograft, autograft and xenograft. Allografts transplant bone or soft tissue from one individual to another in the same species. Allografts have many advantages, including osteoinduction and strong mechanical properties, but carry the risk of disease transmission from the donor, such as HIV, hepatitis or cancer. Autografts, or autologous bone transplants, transplant bone tissue from one site to another in the same individual. This graft offers excellent biocompatibility and does not stimulate host inflammatory response. However, the procedure may cause long lasting pain and discomfort for the patient. Furthermore, there is additional risk of wound infection at the surgery sites [3]. Xenografts remove cells or sections of tissue from one species and graft them on or into a different species. Bovine bone [4] and mollusk shell [5] are common materials used in xenografts. However, the bioactive properties of xenografts are weaker than allografts and autografts. To improve xenografts, a new treatment technique has been introduced for bone or tissue repair called bone tissue engineering – a procedure to regenerate damaged bone by implanting cells, proteins and scaffold to provide mechanical support for gap areas [6]. Bone substitute morphology has many forms, such as a compact and porous structure. As bone replacements, a compact structure provides good mechanical strength while a porous structure is suitable for cell attachment and blood supply [7].

Bone tissue engineering creates a biological material that provides the option for implantation and/or prosthesis. Bone tissue engineering has three main requirements: osteoconductive biomaterial scaffolds, osteogenic cells and osteoinductive molecules [8]. Materials widely used in bone tissue engineering include chitosan, gelatin and HA. Chitosan is a polysaccharide that can be synthesized from crustacean shell and squid pen. Chitosan's structure is similar to glycosaminoglycans, the major component of the extracellular matrix of bone and cartilage [9, 10]. Gelatin can be obtained by thermal denaturation and chemical degradation of collagen [11], and is known to benefit cell viability [12]. In the meantime, HA has a chemical composition similar to human mineral tissue and can be synthesized from many natural sources with calcium-based structures, such as bovine bone, mollusk shell and coral [13-14].

Sintering, salt leaching and freeze-drying have been used for fabricating porous scaffold in bone tissue engineering. Sintering uses high temperatures to bond substances together as well as burn out organic material to form a porous structure [15]. Salt leaching is also feasible, but is limited by the prolonged contact of particles with water and the requirement for salt removal [16]. This study has chosen freeze-drying because it can form highly a porous structure and offer stability and ease of handling [17-18].

This study focuses on preparing and characterizing composite scaffold synthesized from three natural-based materials – chitosan, gelatin and HA. In combination, these three biomaterials offer potential synergies between physical properties and bioactivity for use as bone substitutes in bone grafts, benefiting a range of surgical applications.

II. Materials and Methods

A. Materials Preparation

HA in this study was synthesized from mollusk shell using a wet chemical precipitation method. Mollusk shells were calcined at 850 °C for 5 hours and then ground for 12

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hours to obtain $CaCO_3$ powder. $CaCO_3$ was dissolved in ammonium di-hydrogen phosphate ($NH_4H_2PO_4$) solution and the pH was adjusted by phosphoric acid (H_3PO_4) before precipitating the solution at room temperature for 12 hours to achieve HA with the appropriate Ca/P ratio of 1.0/0.5. Finally, the slurry was calcined at 800 °C for 12 hours to obtain HA powder. Chitosan derived from squid pen extraction was purchased from Taming Enterprises Co., Ltd, Thailand. Gelatin, ammonium di-hydrogen phosphate, sodium hydroxide (NaOH) and phosphoric acid were purchased from Sigma Aldrich Co., Ltd.

B. Preparation of Chotosan-Gelatin Solution

Chitosan solution was prepared by mixing 97.88% deacetylation chitosan with 1% acetic acid solution. Then, gelatin was added to the chitosan solution in a 1:1 ratio and the solution was agitated at 37 °C for 12 hours to form a chitosan-gelatin solution.

C. Fabrication of Chitosan-Gelatin/Hydroxyapatite Porous Scaffold

To develop a porous bone scaffold for this study, HA was added to the chitosan-gelatin solution and stirred for 24 hours to disperse thoroughly. The resultant solution was transferred to well culture plates and pre-frozen at -20 °C for 12 hours, followed by freeze-drying in lyophillizer at -40 °C for 48 hours to obtain porous scaffolds. Then the scaffolds were neutralized by 2% sodium hydroxide and washed with deionized water before repeating the freeze-drying process. A mixture design technique was used to investigate the effect of chitosan-gelatin/HA compositions on scaffold properties. The mixtures were varied based on previous research [18], with a chitosan-gelatin range of 0.50 to 4.00 (%w/w), an HA range of 1.00 to 3.00 (%w/w) and a 1% acetic acid solution range of 93.00 to 98.50 (%w/w).

D. Scaffold Characterizations

The prepared materials and resultant structures of the porous bone scaffolds were characterized for their composition, biodegradability, morphology, porosity, swelling and biocompatibility.

X-ray diffraction analysis (XRD)

X-ray diffraction analysis (XRD) was used to identify the phase of the synthesized HA. The data was collected from a Bruker D8 Advance, using Cu Kα radiation. Voltage and current were set at 40 kV and 40 mA, respectively.

Biodegradability

Biodegradability of the scaffolds was characterized by in vitro study. The scaffolds were immersed in PBS medium containing lysozyme (10,000 U/ml) at 37 °C for 7 days. The initial scaffold weight was denoted as W_0. After 7 days, the scaffolds were washed in deionized water to remove ions adsorbed on the surface and then freeze-dried. The dry weight of the scaffold was denoted as W_t. The degradation of the scaffold was calculated using equation (1).

$$\text{Biodegradability (\%)} = \frac{W_0 - W_t}{W_t} \times 100 \qquad (1)$$

Pore Morphology

Pore structure morphology was examined by scanning electron microscope analysis (SEM). The scaffolds were sputter coated with gold before examining with a JBM-410LV SEM.

Porosity

The porosity of the chitosan-gelatin/HA scaffolds at different concentrations can be determined by Archimedes' principle. Ethanol was selected as the displacement liquid as it permeates the scaffolds without swelling or shrinking the matrix. The dry weight of scaffolds was denoted as W_d, while W_l denoted the weight of the scaffold after immersed in the ethanol for 5 min. Then, the scaffolds were removed and the liquid on the surface removed by filter paper. The weight of the wet scaffold was denoted as W_w. The porosity of the chitosan-gelatin/HA scaffold was calculated using equation (2).

$$\text{Porosity (\%)} = \frac{W_w - W_d}{W_w - W_l} \times 100 \qquad (2)$$

Swelling Ability

Swelling ability was determined by the percentage of water absorption. Dry weight of the scaffold was denoted as W_0. Then, porous scaffolds were immersed in PBS buffer solution with pH 7.4 at 37°C for 24 hours. Afterward, the scaffolds were taken out from PBS buffer solution and its wet weight was measured, denoted as W_w. The ratio of swelling was calculated using equation (3)

$$\text{Swelling ability (\%)} = \frac{W_w - W_0}{W_0} \times 100 \qquad (3)$$

III. RESULTS AND DISCUSSION

A. Materials Characterization

The HA powder synthesized from mollusk shell was characterized using X-Ray diffraction. Figure 1 shows the diffractogram of the synthesized HA, which resembles the naturally occurring bone apatite with respect to degree of crystallinity and structural morphology.

B. Fabrication of Chitosan-Gelatin/Hydroxyapatite Porous Scaffold

The ratio of chitosan-gelatin/hydroxyapatite was varied by the mixture design technique. Physical properties of the developing scaffold from each condition were characterized and recorded, as shown in Table 1.

C. Scaffold Characterizations

Biodegradability, Porosity and Swelling

To investigate the degradability of the structure, the porous scaffold was soaked in PBS solution with lysozyme for 7 days before calculating this property using equation 1. The results showed that when the chitosan-gelatin concentration increased, the scaffold porosity decreased and when the HA concentration increased, both the degradability and porosity of the scaffold decreased. On the other hand, swelling was proportional to the chitosan-gelatin concentration, but inversely proportional to the HA concentration in the mixture. For bone grafting, a porous scaffold should have low biodegradability to allow bone regeneration, while the ideal porosity should be 90.5% to provide the optimal balance between a better surface area for cell attachment and its structural strength [20]. Furthermore, the expansion rate, or swelling ability, of the

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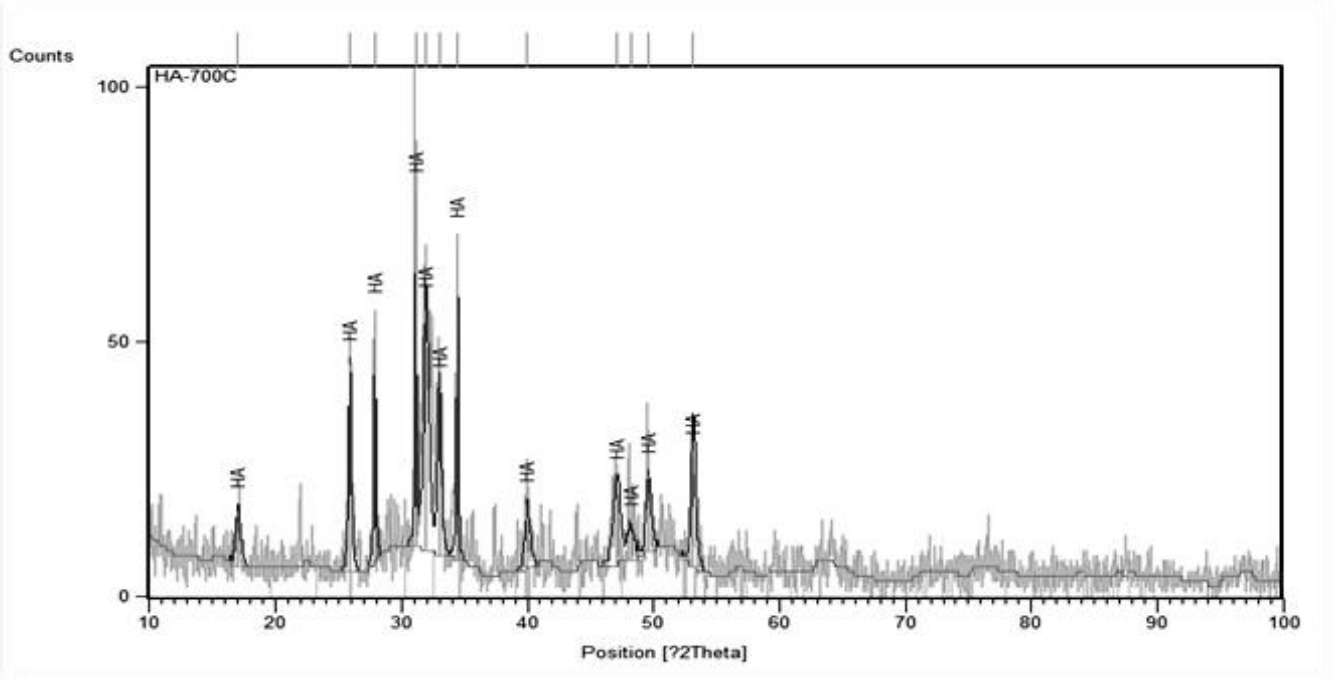

Fig 1. XRD pattern of HA synthesized from mollusk shell

TABLE I
EXPERIMENTAL DESIGN AND RESULTS OF CHITOSAN-GELATIN/HA POROUS SCAFFOLD

Chitosan-Gelatin concentration (%w/w)	HA (%w/w)	1% Acetic acid Solution (%w/w)	Biodegradability (%)	Porosity (%)	Swelling (%)
1.37	1.50	97.13	44.23	91.46	93.24
3.13	2.50	94.37	20.87	89.37	94.32
2.30	2.00	95.70	23.94	92.59	93.10
0.50	1.00	98.50	56.32	94.65	90.76
4.00	3.00	93.00	18.12	88.56	94.15
1.37	1.50	97.13	43.52	91.42	92.45
0.50	3.00	96.50	31.22	90.15	80.45
4.00	1.00	95.00	35.50	92.84	96.88
4.00	3.00	93.00	17.65	88.76	94.23
3.13	1.50	95.37	38.45	91.95	95.66
1.37	2.50	96.13	28.78	89.88	89.66
0.50	3.00	96.50	30.84	90.15	81.89
1.37	2.50	96.13	28.64	89.84	88.35
3.13	2.50	94.37	21.06	89.59	94.48
0.50	1.00	98.50	55.96	94.55	89.74
3.13	1.50	95.37	38.45	91.74	95.45
2.30	2.00	95.70	24.82	91.86	92.95
4.00	1.00	95.00	35.45	92.32	95.45

scaffold is important when employed in a large area; the faster the better. Based on statistical evaluation, the chitosan-gelatin:HA:1% acetic acid ratio of 2.62:2.17:95.21 achieved optimal biodegradability, porosity and swelling properties of 25.6%, 90.5% and 95.5%, respectively.

Pore Morphology

Figure 2 and 3 illustrate the morphology of the fabricated porous scaffold. The morphology of this specimen significantly displays open pore structure with both micropores and macropores. An open pore structure is good for blood supply and cell attachment [12]. The pore size of

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the chitosan-gelatin/HA composite scaffold varied from 50 to 350 μm, as measured by SEM. With increasing concentration of chitosan-gelatin, the pore size decreased, which is in accordance with a previous report [21] and ASTM F2450-10.

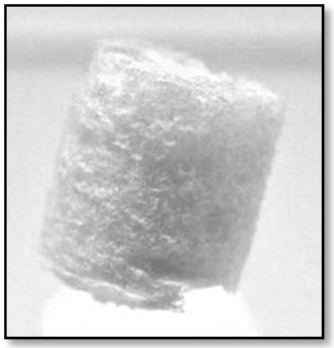

Fig 2. Porous scaffold specimen

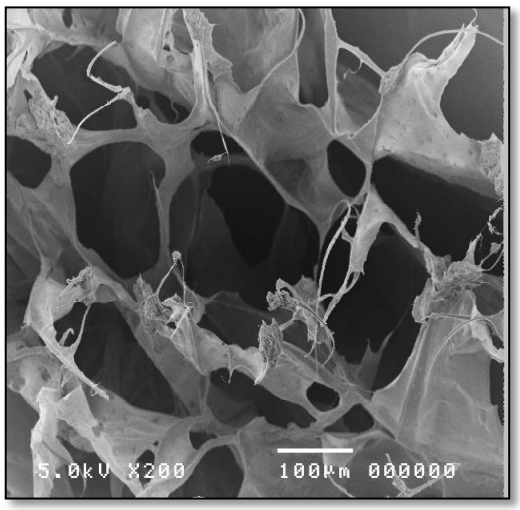

Fig 3. Porous scaffold composite with an open pore structure

IV. CONCLUSION

This study sought to identify a proper mixing ratio between chitosan, gelatin and HA for developing porous bone scaffold that could yield appropriate biodegradability, porosity and swelling properties for bone grafting based on bone tissue engineering technique. The results showed that degradability of scaffold was decreased with increasing chitosan-gelatin and HA concentrations. Porosity increased with decreasing chitosan-gelatin and HA concentrations. Swelling increased with increasing chitosan-gelatin and decreasing HA concentrations. The optimal condition was obtained from the scaffold with a chitosan-gelatin:HA:1% acetic acid ratio of 2.62:2.17:95.21. Furthermore, SEM technique showed an open pore structure of scaffolds – appropriate for blood supply and cell attachment. The porous bone scaffold mix developed here could be a promising bone substitute for bone grafting in the near future.

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