Synthesis of Self-Assembled Hydroxyapatite Membrane Using Bio-Template with Surface Modification

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Abstract:*Many researches in tissue engineering have been conducted continuously to develop the proper synthetic materials for tissue and organ substitute materials. At this research, the synthesis of hydroxyapatite membrane for bone scaffold application has been done successfully by self assembly method using eggshell membrane with surface modification as a template. By this method, the pores obtained will be able to resemble the cells microenvironment thus can enhance interface interaction and support cell proliferation. Before incubating in modified Simulated Body Fluid (SBF) Kokubo solution, eggshell membrane surface is modified using alginate and chitosan solutions to increase the number of hydroxyapatite particles bonded due to enhance the bioactivity of scaffold. The Scanning Electron Microscopy (SEM) result showed that hydroxyapatite membrane has been formed on scaffold's surface where the diameter of fibers had thickened to 2.035, 2.591 and 1.408 μm and particle size of 1.021, 0.934 and 0.895 μm for 6, 9 and 12 days incubation time respectively. It confirmed that the number of hydroxyapatite grown increases along with the incubation time. These results are in good agreement with FT-IR analysis that indicates the presence of PO43- functional group on the scaffolds.*

Keywords: *Eggshell membrane, hydroxyapatite, scaffold, self assembly, and simulated body fluid.*

INTRODUCTION

The need of biomaterials all over the world increases every year. The global biomaterials market is estimated to reach USD 88.4 billion by 2017 from USD 44.0 billion in 2012, growing at a CAGR of 15% [1]. There are some factors that affect the increase of biomaterials need, e.g. the high number of road and work accident, osteoporosis cases, born defect, etc.

Bone loss and defects are frequently encountered in clinics. Several methods such as bone substitutes of autografts, allografts, and xenografts have been applied in clinics to repair the bone defects [2]. However, these bone substitutes have some limitations in the application such as the availability of donor sites, antigenicity issues, post surgery suffer and trauma, and the high cost.

To overcome these problems, in the past decades, many active researches in the field of tissue engineering have been conducted continuously and some scientists and engineers are trying to mimic the natural processes and designs to develop the proper synthetic materials for bone scaffolds. According to Kim et al. [3], to bring about the desired biological response, ideal scaffolds should be highly porous with an interconnected pore network and flow transport of nutrients and metabolic waste. Furthermore, they must be biodegradable with controllable degradation, have both a suitable surface chemistry for cell attachment and the appropriate mechanical properties, and be easily processed to form a variety of shapes and sizes.

Natural and synthetic polymers that can be applied for bone scaffolds are collagen, chitosan, alginate, poly (εcaprolactone), poly lactic acid (PLA), polyhydroxybutirate-co-hydroxyvalerate (PHBV), etc. Another natural polymer that can be found almost everywhere and in large quantities as an industrial waste natural product is eggshell membrane. Eggshell membrane consists mainly of proteins such as collagen (type I, V, and X), osteopontin, and sialoprotein [4]. Some researches reported that eggshell membrane has an interconnected porous structure [3-5], biocompatible [3,4,6], and biodegrable. Chitosan is the deacetylated derivative of chitin which is the second most abundant polysaccharide isolated from crustaceans (crab, shrimp, etc). Chitosan has relatively good mechanical properties, biocompatibility, biodegradability, and non-toxicity. Chitosan has been used to improve the mechanical properties of other natural polymers such as keratin, silk, and starch [6]. Alginate is abundant in nature and found as structural components of marine brown algae and capsular polysaccharides in some soil bacteria [7]. Alginate has interconnection and open pore structure, high biocompatibility, and biodegradability. Alginate has been used for pharmaceutical applications like wound dressings, dental impression materials, cell encapsulation, etc. It can also be used to modify substrate surface to be more flexible.

Other important ones are bioactive ceramics such as calcium phosphates and bioactive glasses. The calcium phosphate compounds including hydroxyapatite, α-tri calcium phosphate (α-TCP), and β-tricalcium phosphate (βTCP) are more extensively applied as the bone substitutes [2]. Hydroxyapatite is a major constituent of bone which has osteoconductivity and can be biodegradable [8]. However, to overcome its brittleness and poor processing property, more recently, attention has been paid to the composites of polymers and ceramics, with the aims of increasing the mechanical stability and improving tissue interaction [2].

In this work, biocomposite scaffold is produced with eggshell membrane as a template and a support by self assembly method. By this way, the pores obtained will be able to resemble the cells microenvironment thus can enhance interface interaction and support cell proliferation. Surface modification is conducted using alginate to improve the flexibility of the template and chitosan to increase the mechanical strength and increase the number of hydroxyapatite particles bonded due to enhance the bioactivity of scaffold.

MATERIALS AND METHODS

Materials

The eggs were purchased from local grocery. Raw eggshell membrane was obtained by peering manually from commercial eggshells. All chemicals and reagents were purchased from commercial sources and were used without further purification. Sodium alginate (from brown algae), chitosan (low molecular weight, 75 – 85% deacetylated), and inorganic salts (NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, $CaCl_2$, and Na_2SO_4) for production of modified simulated body fluid (SBF) Kokubo solution were purchased from Sigma-Aldrich. Demineralized and deionized water were obtained from local supplier.

Methods

Preparation of eggshell membrane. The eggs obtained from local grocery were carefully broken and the contents were emptied. The eggshells were repeatedly washed in water and the inner eggshell membrane was removed manually. The eggshells with the outer eggshell membrane were dipped in 1 M nitric acid solution to dissolve the calcium carbonate shell. The undissolved outer eggshell membrane was rinsed several times with demineralized water and then immersed in NaOH solution to remove the non-collagenous protein and to activate its surface.

Preparation of modified SBF Kokubo solution. SBF is a solution which has inorganic ions concentration similar to those of human blood plasma (see Table 1), in order to reproduce formation of apatite on bioactive materials under biomimetic condition. At this research, the modified SBF Kokubo solution consists of inorganic ions with concentration of 1.5 times of human blood plasma. This solution was prepared by dissolving NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$, $CaCl_2$, and Na_2SO_4 one by one into deionized water under stirring. The final pH of SBF solution was set to 7.4 using 1 M HCl solution and the temperature was held at 36.5 °C [9].

TABLE 1: Ion concentrations of SBF Kokubo and human blood plasma

Ion	Concentration [mM]	
	SBF Kokubo	Human blood plasma
Na^+	142,0	142,0
K^+	5,0	5,0
Mg^{2+}	1,5	1,5
Ca^{2+}	2,5	2,5
Cl^-	147,8	103,0
HCO_3^-	4,2	27,0
HPO42–	1,0	1,0
SO42–	0,5	0,5

Synthesis of hydroxyapatite membrane. After immersing of eggshell membrane in NaOH solution, it was washed using demineralized water. Subsequently, the membrane was immersed in 1% ($^w/_v$) alginate solution and 1% ($^w/_v$) chitosan solution consecutively. The membrane was then incubated in modified SBF Kokubo solution to produce hydroxyapatite layer on its surface by self assembly method. The incubation time was varied for 6, 9, and 12 days. The SBF solution was refreshed periodically during incubation.

Characterizations. The scaffold obtained after incubation process was characterized by Scanning Electron Microscope (SEM) and Fourier Transform Infra Red (FT-IR). SEM (type JEOL-JSM 6360 LA) was used to observe the microstructure of the scaffold and FT-IR (type A FT/IR 4200) was used to investigate the functional groups contained on the scaffold. The FT-IR spectrum was recorded over a wave number range of 400 – 4000 cm^{-1}.

RESULTS AND DISCUSSION

The microstructure of outer eggshell membrane is showed in Fig. 1. The membrane is composed of macroporous network of interwoven and coalescing membrane fibers with diameters ranging from 0.5 – 2 μm. After activation treatment by immersing in NaOH solution, there is a quite difference on the surface where the mesh seems to be cleaner. It may possibly happen because NaOH treatment will also clean the membrane from non-collagenous protein.

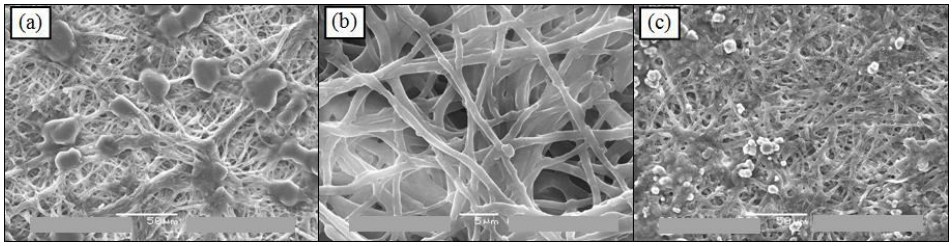

Fig 1: Microstructure of eggshell membrane: (a) before immersing in NaOH solution; (b) Fig. 1a with higher magnification; (c) after immersing in NaOH solution.

Modification of eggshell membrane surface was carried out by immersing the membrane in alginate and chitosan solution consecutively. Alginate was employed to introduce the flexibility of the scaffold whereas chitosan layer was used as surfactant. The activated membrane was incubated in modified SBF Kokubo solution to generate hydroxyapatite on its surface. Fig. 2 shows SEM images of scaffold after 6, 9, and 12 days incubation time. The figure denotes that the fiber had thickened along with the incubation time to 2.035, 2.591, and 1.408 μm for 6, 9, and 12 days incubation time respectively. This indicates that alginate, chitosan, and apatite are already existed on the surface. The presence of chitosan will accelerate the apatite bonded on the surface. Amine group from chitosan has polar characteristic so that it will be easier to bind OH^- group from modified SBF Kokubo solution. The greater of OH^- ions bonded on the surface, the greater of Ca^{2+} and PO_4^{3-} ions that will bind OH^- ions to form apatite.

Incubation process in modified SBF Kokubo solution at certain time periods affects the swelling of the biopolymers which is followed by biodegradation process. It occurs when the scaffold was incubated in 6 and 9 days where the increase of fiber diameter is occurred significantly compare to raw eggshell membrane. After 12 days incubation time, the fiber diameter of eggshell membrane decreased, indicating the occurrence of biodegradation of natural polymer. It is also showed by the color change of SBF solution from transparent that becomes blurry when the scaffold is immersed for longer time although the exchange of SBF solution is performed periodically.

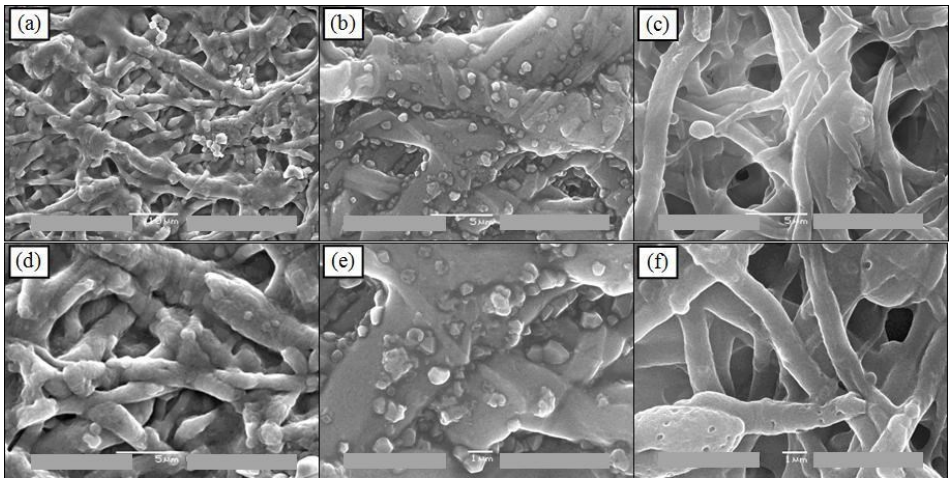

Fig 2: SEM images of scaffold after incubation process in modified SBF Kokubo solution for: (a) 6; (b) 9; and (c) 12 days.

[Fig. 2d, 2e, and 2f are the high magnification of Fig. 2a, 2b, and 2c respectively]

The SEM images of scaffold in Fig. 2 demonstrates that the hydroxyapatite has been grown successfully on membrane surface by self assembly with the particle size of 1.021, 0.934, and 0.895 μm respectively after incubation time of 6, 9, and 12 days in SBF solution. These results are in good agreement with FT-IR analysis (Fig. 3) after incubation process that indicate the presence of PO_4^{3-} functional group on the scaffolds at wavenumber 559.255 and 1087.66 cm^{-1}.

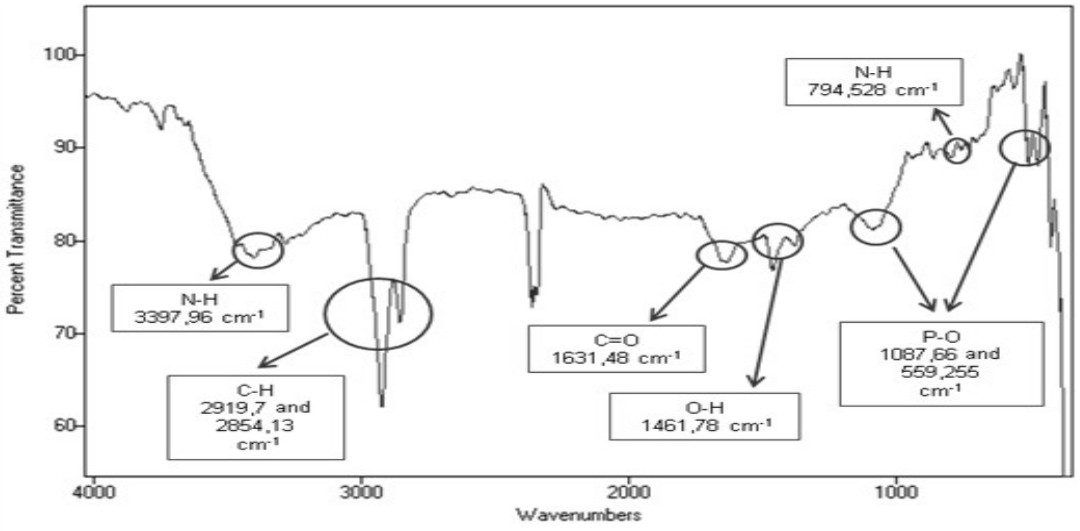

Fig 3: FT-IR spectrum of scaffold after incubation in modified SBF kokubo solution.

The mechanism of apatite formation on membrane surface can be explained briefly as follow: when the membrane is incubated, the apatite will start growing. OH^- functional group from SBF solution, alginate, chitosan, and eggshell membrane will gather on its surface. It is possible because the OH^- group is hydrophilic. The presence of OH^- ion on the surface will make the Ca^{2+} ion, which has positive charge, bonded by OH^- group and followed by PO_4^{3-}. The system of OH^-, Ca^{2+}, and PO_4^{3-} is called apatite. It is showed from the SEM result that the scaffold which is incubated for 12 days will produce the apatite with larger amount compare to the others. Longer incubation time enable the greater amount of apatite to grow as the apatite source (SBF solution) is refreshed periodically.

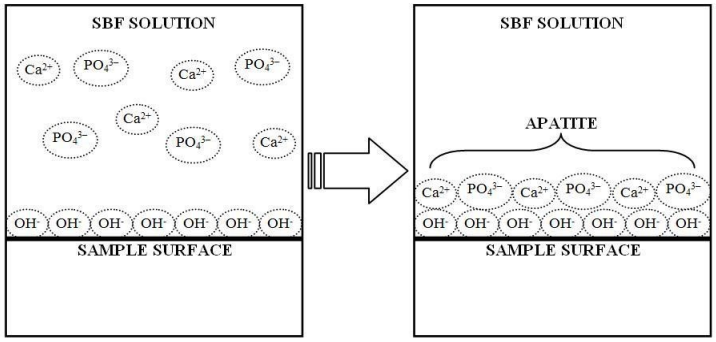

Fig 4: Illustration of apatite formation on membrane surface.

CONCLUSION

The synthesis of hydroxyapatite membrane for bone scaffold application has been demonstrated by self assembly method using eggshell membrane with surface modification as a template. The longer of incubation time in modified SBF Kokubo solution affected the greater amount of hydroxyapatite grown on its surface with smaller particle size.

The presence of hydroxyapatite was indicated by PO_4^{3-} functional group contained in all samples after incubating. Incubation in certain time period indicated the degradation process of natural polymer materials used. The process was occurred significantly by the increase (swelling) and subsequently the decrease of fiber thickness.

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