Preface

Wilujeng Sumping,

Welcome to Bandung to the International Conference on Instrumentation, Communication, Information Technology and Biomedical Engineering (ICICI-BME) 2011.

On behalf of the organizing committee, we are delighted to welcome all of the participants to the ICICI-BME 2011. This biennial conference is organized under the auspices of the Institut Teknologi Bandung (ITB), Indonesian Sensor and Actuator System Society (ISASS), Indonesian Biomedical Engineering Society (IBES) and sponsored by the Faculty of Mathematics and Natural Sciences, School of Electrical Engineering and Informatics, Faculty of Industrial Technology, Faculty of Mechanical and Aerospace Engineering of ITB and IEEE Engineering in Medicine and Biology Society (EMBS).

ICICI-BME is dedicated to the presentation and discussion of the latest developments and ideas in instrumentation, measurements, communication, information technology and biomedical engineering, in both theory and application.

This conference also aims to strengthen the collaboration among international researchers, scientists, engineers and industrial players in the fields of science and engineering. It is designed to be a meeting point for those who are involved, to globally exchange and share their views, ideas and advances in science, technology, and industrial aspects.

My gratitude to many people which helped making this conference a reality, to all of our invited speakers and guests, and for all of our committee members for their effort to ensure the success of this conference. Finally, I hope that all of participants will learn new things, make new contacts, get new ideas and have fruitful discussion while having a pleasant experience during our conference in Bandung.

Hatur nuhun, Thank you

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The Effect of Sericin Application Over Hydroxyapatite Surface on Osteoblast Cells Proliferation

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Abstract- Hydroxyapatite (HA) has been used clinically to treat bone defect. Modification of HA surface with respect to bone integration has been developed using treatments which have the potential to improve cell proliferation. Bombyx mori's sericin, a polymer protein that has polar side groups, have been known to accelerate cells attachment and proliferation. The aim of this study was to investigate the effect of sericin application over HA surface on osteoblast cells proliferation. HA discs (10 mm in diameter, 3 mm thick) were sintered. Three concentration of sericin (0.01, 0.5, and 0.1%) were applied on HA surface. Water contact angle was measured to evaluate the hydrophilicity of the disc surface. The discs were then seeded with MC3T3E1 osteoblast cells for proliferation test. The data were analyzed by Anova and LSD. Contact angle measurement showed significant increases of the hydrophilicity on sericin-modified HA surface. The amount of cells proliferation on HA discs (1.40±0.26) was significantly different (p<0.05) with HA+sericin 0.01% (2.23±0.20), HA+sericin 0.5% (2.33±0.24), HA+sericin 0.1% (2.37±0.20). Variation of sericin concentrations applied over HA did not influence any significant difference on cells proliferation (p=0.05). The conclusion was sericin application over HA surface increased the amount of osteoblast cells proliferation. Concentration of sericin application over HA (0.01, 0.05, 0.1%) did not influence cells proliferation.

Keyword: hydroxyapatite, sericin, Bombyx mori, proliferation, osteoblast

I. INTRODUCTION

Drawbacks to current repair strategies for patients suffering from bone defects include tissue availability and donor site morbidity. Bone tissue engineering is an emerging technique that offers potential solutions to these problems. Scaffolds may be used to support and encourage cellular activity and promote faster healing. Hydroxyapatite (HA) is a calcium phosphate ceramic that has been used clinically and has been shown to have bioactive, osteoconductive and biocompatible properties. It may be possible however, to further enhance HA with respect to bone integration using treatments that have the potential to improve cell proliferation and thus improve implant integration and wound healing.

The process of cell interactions on materials is highly dynamic and depends on various parameters influencing the cell responses [4]. Cell attachment and proliferation on biomaterials depend on surface characteristic such as wettability (hydrophilicity or hydrophobicity or surface free energy), chemistry, charge, topography and rigidity [4-5]. Amino-, hydroxyl-, carboxyl-, sulfonic-, acylamino- groups favor cell attachment and growth [6]. Positively charged surfaces seem better for cell adhesion, spreading and growth than negatively charged. Polymer surface grafted with amine groups is best for cell adhesion, spreading and growth in aqueous cell culture medium than hydroxy groups and amide groups due to its positively charged [5].

It has been found that osteoblast adhesion strongly correlates with substratum wettability, with high rates of cell attachment on relatively hydrophilic surfaces (contact angle < 65°) and low attachment rates on hydrophobic surfaces (contact angle > 65°) [7]. Carboxylic acid groups also favor cell attachment and proliferation due to the increased of wettability [94].

Sericin, a natural protein derived from arthropods e.g. silkworm Bombyx mori, has recently investigated for its activities in the biotechnological field. Sericin protein is highly hydrophilic and made of 18 amino acids most of which have strongly polar side groups such as hydroxyl, carboxyl and amino groups and characterized by high serine [8]. Several studies showed that sericin supports cell adhesion and proliferation when used in pure form and blended in matrices. Sericin enhances the attachment and growth of mouse fibroblast when used as a substratum as high as collagen [9]. In a dose dependent manner, sericin accelerates proliferation of mammalian cells line in culture. Sericin can be coated on biomaterial surface by chemical reaction using N-(3-dimethylaminopropyl)-N-ethylcarbodiimide (EDC) and N-hydroxysuccinimide (NHS) as coupling agents, and has been shown to enhance functionality in promoting osteoblast adhesion, proliferation, and alkaline phosphatase activity [11].

Although HA has already been applied as tissue engineering material, and sericin has already been known to enhance cells attachment and proliferation, there is no data on the application of sericin over HA surface and its potency to enhance cells proliferation. This study was aimed to investigate the effect of...
sericin application over HA surface on osteoblast cells proliferation.

II. MATERIALS AND METHODS

Materials

The HA used in this study was synthesized from local gypsum (Kulon Progo, Yogyakarta, Indonesia). Sericin was extracted from cocoon shells of the silkworm Bombyx mori which were obtained from PT Yarsilk Goramahottama Textile Industry (Yogyakarta, Indonesia). Osteoblast cells of MC3T3E1 were obtained from Niigata University. Diammonium hydrogen phosphate (DHP), sodium carbonate, NHS, EDC, Phosphate Buffer Saline (PBS), alpha minimum essential medium (alpha MEM), fetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma-Aldrich Chemicals (Bornem, Belgium)

Preparation of HA discs

The synthesis of HA was conducted as Katsuki [12] and Pujianto [13]. The gypsum powder was obtained by pulverizing the gypsum rock. Gypsum powder (20 g) and 800 ml of 1 M DHP were mixed at 100°C for 20 minutes in a Pyrex glass using a microwave digestion system. The system was operated at frequency of 2.45 GHz. After the hydrothermal reaction, reacted sample were washed with distilled water to remove residual ion and then dried. Powder of HA (0.4 g) was put in a mold (10 mm in diameter, 3 mm thick) and compacted with the pressure of 80 MPa for 30 s, followed by sintering at 1300°C for 4 hours with a heating rate of 60°C/h.

Preparation of sericin

The extraction of sericin was done as Zhang [11]. Cocoon shells of Bombyx mori were cut into pieces (1x1 cm). One liter of 0.2% sodium carbonate solution containing 40 g of the cocoon was boiled for 1 hour and then filtered through a glass microfiber filter in order to remove fibroin and other impurities. The filtrate was then dialyzed using membrane cellulose against deionized water for 2 days by changing the water daily to remove the ions and other impurities and then freeze dried at -60°C for 15 hours.

Application of sericin over HA surface

The surface modification method was adopted from Cui [14]. Disc of HA was placed in 1 M NaOH solution at 50°C for 1 hour then rinsed with 0.1 N HCl and distilled water at room temperature. The disc was precipitated for 1 hour at room temperature in PBS solution containing 1 mg/ml of NHS and 10 mg/ml EDC. The substrates were then transferred to PBS solution containing of sericin with different concentrations (0.01, 0.05, and 0.1%). The reaction was allowed to proceed for 6 hours at room temperature. Contact angles were measured with a sessile drop method. A 5 μl water droplet was placed on the disc surface and the static contact angle was measured using digital camera (Canon 30D) with macro lens EF 100 mm 1:2.8. Three measurements on different areas of the surface were obtained for each reported contact angle value.

Cell culture

Mouse osteoblast cell line MC3T3E1 cells were cultured in alpha MEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 5% CO2 and 95% air with the growth medium changed every 48 hours. The cultured cells were detached by trypsinization (0.25% trypsin-EDTA), suspended in fresh culture medium and used for the designed proliferation assay.

Cell proliferation

The proliferation of osteoblast cells on substrates was examined by counting the number of cells after 4 days [15]. The substrates were placed into a 24-well plate and seeded with a density of 2000 cells/cm2. The substrates were rinsed with PBS to remove unattached cells. Adherent cells were then detached from the substrates by trypsin and measured via a hemocytometer.

III. RESULT AND DISCUSSION

In this study, Bombyx mori’s sericin had been used to improve cellular interaction capability of HA surface. Sericin was covalently immobilized using carbodiimide and NHS in three different concentrations (0.01, 0.5, and 0.1%). To covalently immobilized protein molecules in the chemically inert biomaterials, reactive groups, in this case carboxyl, must be firstly introduced as coupling sites. Hydrolysis of HA was done by treating in NaOH solution to produce reactive groups. One problem in hydrolysis process was the molecular weight of material would be partially sacrificed, thus the reaction conditions should be well controlled [16]. When material was immersed in NaOH solution, hydrolysis process produced carboxyl on the material surface. It followed by the activation of the carboxyl group with water soluble carbodiimide. The NHS was added to form more stable amide bonds. Final reaction was between the activated carboxyl groups and the amino groups of sericin that produce amide bond (N-H).

Water contact angle was measured to evaluate the hydrophilicity of the surface materials as in table 1. Measurement of the contact angle on HA and sericin-modified HA surfaces gave an indication of the relative hydrophilicity of these surfaces before and after the modification. Ordinarily, more hydrophilic surfaces showed smaller contact angles value. All of sericin-modified HA groups demonstrated lower contact angle value significantly, implying a great improvement of the hydrophilicity. The contact angle of HA surface was measured to be approximately 87.46° and
decreases to about 42° after modified with sericin. Sericin contains a large amount of amino acids with polar functional groups such as hydroxyl, carboxyl, and amino groups [17]. The strongly polar groups of sericin gave rise to a more wettable surface on sericin-modified HA as indicated by the changes of contact angle. Sericin coated on polystyrene films has increased the hydrophilicity of the films, with contact angle of 84° on polystyrene and 46° on sericin coated polystyrene [9].

<table>
<thead>
<tr>
<th>Table I</th>
<th>MEAN AND STANDARD DEVIATION OF CONTACT ANGLE SURFACE (DEGREE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Contact Angle (Degree)</td>
</tr>
<tr>
<td>HA</td>
<td>87.460 ± 1.040</td>
</tr>
<tr>
<td>HA + Sericin 0.01%</td>
<td>42.200 ± 1.010</td>
</tr>
<tr>
<td>HA + Sericin 0.05%</td>
<td>41.600 ± 1.320</td>
</tr>
<tr>
<td>HA + Sericin 0.1%</td>
<td>41.200 ± 1.120</td>
</tr>
</tbody>
</table>

Statistical analysis using one way Anova of the contact angle data showed that water contact angle of sericin-modified HA had significant lower than that of control HA (p<0.05) (table II). It means that the hydrophilicity of surface-modified HA with all sericin concentrations were greatly enhanced. Sericin with initial concentration of 0.01% had the lowest contact angle, however the hydrophilicity between groups of different sericin concentrations were not significantly different (p>0.05) (table III).

It was generally known that the hydrophilicity of a surface could affect the degree of cell adhesion and proliferation. Hydrophilicity of a material was believed to be a factor affecting the surface energy (surface tension) which might influence serum proteins that adhered to the material, and in turn governed the biological response, such as cell adhesion and proliferation [7]. Although hydrophobic surfaces tended to bind more protein [18], many cell studies had been reported that cells attached and spread more effectively on surface with proper hydrophilicity than on hydrophobic surfaces.

Human fetal osteoblast cell line (hFOB) had high attachment rates on relatively hydrophilic surfaces (contact angle θ<65°) and low attachment rates on hydrophobic surfaces (contact angle θ>65°) [19]. In a study on the interaction of different types of cells (Chinese hamster ovary, fibroblast, and endothelial cells) as well as serum proteins in terms of the surface hydrophilicity of polymeric materials was observed that the cells were adhered, spread, and grown more on the position with moderate hydrophilicity than on the more hydrophobic position. The maximum adhesion and growth of the cells appeared at around water contact angles of 55° regardless of the cell types used [20].

Table II

<table>
<thead>
<tr>
<th>Table II</th>
<th>SUMMARY OF ONE-WAY ANOVA OF WATER CONTACT ANGLE MEASUREMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of squares</td>
<td>df</td>
</tr>
<tr>
<td>Between groups</td>
<td>3989.995</td>
</tr>
<tr>
<td>Within groups</td>
<td>22.335</td>
</tr>
<tr>
<td>Total</td>
<td>4012.330</td>
</tr>
</tbody>
</table>

Table III

<table>
<thead>
<tr>
<th>Table III</th>
<th>SUMMARY OF LSD TEST OF WATER CONTACT ANGLE MEASUREMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups Treatment</td>
<td>Mean Difference</td>
</tr>
<tr>
<td>HA versus HA + Sericin 0.01%</td>
<td>36.175</td>
</tr>
<tr>
<td>HA versus HA + Sericin 0.05%</td>
<td>36.675</td>
</tr>
<tr>
<td>HA versus HA + Sericin 0.1%</td>
<td>36.550</td>
</tr>
<tr>
<td>HA + Sericin 0.01% versus HA + Sericin 0.05%</td>
<td>0.200</td>
</tr>
<tr>
<td>HA + Sericin 0.01% versus HA + Sericin 0.1%</td>
<td>0.375</td>
</tr>
<tr>
<td>HA + Sericin 0.05% versus HA + Sericin 0.1%</td>
<td>0.125</td>
</tr>
</tbody>
</table>

The cell studies was carried out to investigate the proliferation of osteoblast cells on sericin-modified HA discs. Table IV showed the number of cells proliferation after 4 days incubation. The result showed that the application of sericin over HA surface increased the amount of cells proliferation. Higher sericin concentration over HA surface tended to increase the amount of osteoblast cells proliferation. Statistical analysis of the data on cells proliferation was as table V.

Table IV

<table>
<thead>
<tr>
<th>Table IV</th>
<th>MEAN AND STANDARD DEVIATION OF OSTEOBLAST CELLS PROLIFERATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Material</td>
<td>Proliferation (x10^3)</td>
</tr>
<tr>
<td>HA</td>
<td>1.400 ± 0.260</td>
</tr>
<tr>
<td>HA + Sericin 0.01%</td>
<td>2.230 ± 0.200</td>
</tr>
<tr>
<td>HA + Sericin 0.05%</td>
<td>2.330 ± 0.240</td>
</tr>
<tr>
<td>HA + Sericin 0.1%</td>
<td>2.370 ± 0.200</td>
</tr>
</tbody>
</table>

Table V

<table>
<thead>
<tr>
<th>Table V</th>
<th>SUMMARY OF ONE-WAY ANOVA OF OSTEOBLAST CELLS PROLIFERATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sum of Squares</td>
<td>df</td>
</tr>
<tr>
<td>Between Groups</td>
<td>1.4900</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.520</td>
</tr>
<tr>
<td>Total</td>
<td>2.420</td>
</tr>
</tbody>
</table>

Table V showed that there was a significant difference (p<0.05) among the sericin application over HA surface on cells proliferation. To determine later about the effect of sericin concentration, it was performed the LSD test as in table VI.

Table VI

<table>
<thead>
<tr>
<th>Table VI</th>
<th>SUMMARY OF LSD TEST OF OSTEOBLAST CELLS PROLIFERATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Groups Treatment</td>
<td>Mean Difference</td>
</tr>
<tr>
<td>HA versus HA + sericin 0.01%</td>
<td>0.830</td>
</tr>
<tr>
<td>HA versus HA + sericin 0.05%</td>
<td>0.930</td>
</tr>
<tr>
<td>HA versus HA + sericin 0.1%</td>
<td>0.970</td>
</tr>
<tr>
<td>HA + sericin 0.01% versus HA + sericin 0.05%</td>
<td>0.100</td>
</tr>
<tr>
<td>HA + sericin 0.01% versus HA + sericin 0.1%</td>
<td>0.130</td>
</tr>
<tr>
<td>HA + sericin 0.05% versus HA + sericin 0.1%</td>
<td>0.030</td>
</tr>
</tbody>
</table>
Table VI showed that there were significant differences (p<0.05) between the amount of osteoblast cells proliferation on HA surface versus sercin-modified HA surface in all sercin concentrations application (0.01, 0.05, 0.1%). There were no significant differences (p>0.05) between the amount of osteoblast cells proliferation on sercin-modified HA surface on different concentrations.

The proliferation of osteoblast cells on HA and sercin-modified HA after 4 days were compared. All of the sercin modified HA showed higher cell numbers than that of control HA, but there were no significant differences between three different concentrations of sercin. Although the cell numbers were significantly different, sercin modified HA did not show an increasing different proliferation.

Cells proliferation correlated with substratum surface wettability and functional groups on the surface. Moderately hydrophilic surface were shown better cells growth compatibility for fibroblast, Chinese hamster ovary cells and endothelial cells after 2 days of culture [20]. The hydrophilicity and functional groups of PLLA modification with chitosan also related with higher proliferation of chondrocytes [21], fibroblast [22] and osteoblast like cells [7]. Functional groups such as amine, hydroxyl and carboxyl also compatible for cell growth, and the behavior was similar to cell adhesion [5].

After attached on substratum, cells will undergo a progressive process of morphological changes and spreading before proliferate. This sequence of events was delayed and attenuated on poorly-cytocompatible hydrophobic substrata. Poorly cyto compatible surfaces exhibit characteristically-low attachment efficiency and long induction periods during which cells apparently engaged in a life or dead struggled to improve the pericellular environment by excretion of matrix [23]. Proliferation itself is a dynamic process regulated by cell adhesion as subsequent phase of attachment. The binding of ECM molecules to integrin receptors causes changes in the cytoskeleton that ultimately affect gene expression in the nucleus. Cell process receptors for growth factors that can act through a cascade of intracellular factors to alter gene expression that affect cell proliferation [18]. It explained that proliferation depends on the survival of cell attachment to a substratum.

Sercin significantly increased the cell proliferation when mixed with culture medium at concentration of 0.03 – 0.1%. Lower concentration did not show significant increasing in cell proliferation. However, higher concentration was severely harmful to the culture [10]. The result of this study indicated that the amount of immobilized sercin on HA surface was probably below the efficient concentration to enhance osteoblast proliferation. However, in this study, the amount of immobilized sercin could not be determined. As mentioned before, the amount of immobilized sercin depended on the amount of preactivated carboxyl groups created by hydrolysis process. This study was using one concentration of NaOH (0.1M) to create the carboxyl group on HA surface. The similar proliferation rate indicated that carbodiimide chemical reaction between carboxyl groups of HA and sercin with three different concentrations (0.01, 0.05, and 0.1%) resulted in the similar amount of immobilized sercin on HA surface but still below the amount needed to enhance cell proliferation.

Other concern of the covalently protein immobilization is that the natural conformation of the grafted protein might be changed [16]. Studies by Terada [10], Tsubouchi [24], and Minoura [9] used sercin in direct application without chemical reaction. In this study, the immobilization of sercin using carbodiimide chemistry might change the natural conformation of sercin protein which might slightly degrade sercin properties to enhance cells proliferation. The increase in cell count observed after 4 days of culture was due to the cell count. Since cell proliferation was occurred as subsequent phase after 24 hours of adhesion, the increases in cell count were to be attributed to enhanced initial cell attachment not to the sercin properties in accelerating cell proliferation.

The result of this study showed that the application of sercin over HA by concentrations of 0.01 – 0.1% did not differentiate cell proliferation. The wider range of sercin concentration to modify HA with carbodiimide chemistry needed to be determined to know the efficiency of sercin concentration to enhance osteoblast cells proliferation. The hydrolysis process to create preactivated carboxyl groups was also an important factor to be observed because it determined the final concentration of sercin that bound to HA surface.

For tissue engineering application, sercin-modified HA had to fulfill the requirement as an ideal scaffold material that compatible for the implanted cells. Therefore, more data about osteoblast cells behavior on sercin-modified HA such as its differentiation into specific cell phenotype would be needed. However, this research had contributed to show the initial cell response on sercin and to give alternative for surface modification of HA.

III. CONCLUSION

The conclusion of this study was that sercin application over HA surfaces increased the amount of osteoblast cells proliferation. Concentration of sercin application over HA (0.01, 0.05, and 0.1%) did not influence cells proliferation. Further suggestion related to this study was that further research would be needed especially on the wider range of sercin concentration to modify HA surface to observe the efficiency of sercin concentration to enhance cells proliferation. The hydrolysis process to create preactivated carboxyl groups was also an important factor to be observed because it determined the final concentration of sercin that bound to HA surface with regard to its effect on HA mechanical properties.

REFERENCES


