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MATERIALS AND METHODS

Place and Time Schedule

This research was being conducted from February through September 2010 which took place in IPB-Biophysics Laboratory in sample preparation, while *in vitro* sample analysis was done in Oral Biology Laboratory of Dental Faculty, University of Indonesia. Sample sterilization was done in National Nuclear Energy Agency (BATAN) Pasar Jumat. Sample characterization was done in National Nuclear Energy Agency (BATAN) Serpong for SEM characterization.

Materials and Equipments

Materials

1. BCP synthesis materials :
   a. Calcium oxide from chicken eggshell
   b. Pro-analyze ammonium hydro phosphate; $(NH_4)_2HPO_4$
   c. Aquabides
2. MG-63 cell line as osteoblast cell
3. Cell culture medium materials :
   a. Dulbecco's Modified Eagle's Medium (DMEM)
   b. Fetal Bovine Serum (FBS)
   c. Penicillin Streptomycin
   d. Fungizone
4. Trypsin EDTA
5. Trypan blue
6. Washing medium materials : Phosphate Buffered Saline (PBS)
7. Cytotoxicity test materials :
   a. MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide)
   b. Acidified Isopropanol
   c. NaCl solution

8. SEM preparation materials :
   a. Phosphate Buffered Saline (PBS)
   b. 8% glutaraldehyde
   c. Ethanol

Equipments

1. BCP and HA synthesis equipments (Appendix 4) :
   a. Analytical balance
   b. Furnace
   c. Heating plate
   d. Hydrothermal reactor
   e. Burette
   f. Vacuum
   g. Digital pHmeter
   h. Beaker glass
   i. Crucible
   j. Mortar
   k. Filter paper
   l. Magnetic stirrer
   m. Aluminum foil
   n. Petri plate
2. Gamma radiation sterilization with cobalt 60-radiation source (Appendix 6)
3. In vitro analysis equipments (Appendix 5) :
   a. 0.2 μm-sterile syiringe filter (Corning, Germany)
   b. 50 mL-syringe (Terumo, Japan)
   c. 15 mL- and 50 mL-tube (Falcon, USA)
   d. Scrapper
   e. Micropipette (Eppendorf, Germany)
   f. Tips micropipette
   g. Tube eppendorf (Axygen, USA)
   h. Hemocytometer

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i. Incubator (Memert)
j. Cell culture dish (35 mm×10 mm)
k. 96-well plates (NUNC, Denmark)
l. Microscope (Nikon Elipse 80i)
m. Biohazard safety cabinet (ESCO Micro PTE Ltd.)
n. Water bath
o. Centrifuge (Sorvall)
p. Vortexer (Bio-rad BR 2000)
q. Shaker (Certomat)

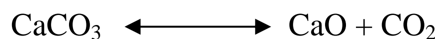
4. Characterization equipments (Appendix 6) :
   a. Scanning Electron Microscope (SEM) (JEOL JCM-35C)
   b. Ion Sputter JFC-1100 machine
   c. Bio-Rad Microplate Reader Benchmark Visible Spectrophotometer

Experimental Method

Experimental method is shown on flow chart on Appendices 1, 2, and 3.

Hydroxypatite synthesis

The same raw materials of CaO as synthesizing BCP that gained from chicken eggshells were used to prepare 100 mL-CaO suspension which was seen as white thick fluid. Chicken eggshells that mostly contain $CaCO_3$ were calcined at 1000°C for 5 hours as chemical reaction below:



$$CaCO_3 \longleftrightarrow CaO + CO_2$$

0.3 M CaO suspension of eggshell product was dropped by 0.18 M of clear $(NH_4)_2HPO_4$-solution in 100 mL aquabides in 37°C while stirring at 300 rpm at the rate of 7 mL/min. The final suspension was then be filtered under vacuum. The filtered cake was then being dried in the furnace at 110°C during 5 hours. The dried powder was then be sintered at 900°C during 5 hours by using furnace. The chemical reaction of CaO and $(NH_4)_2HPO_4$ was as below:

$$10CaO + 6(NH_4)_2HPO_4 + 14H_2O \longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 12NH_4OH + 10H_2O$$

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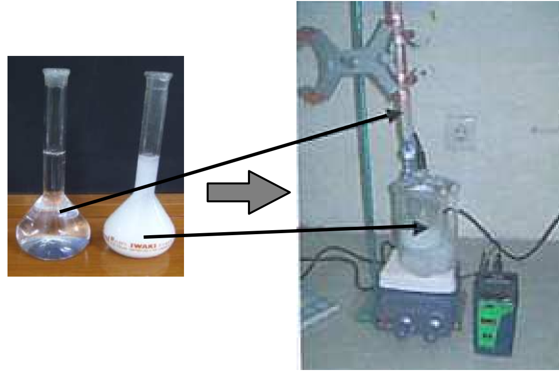

Figure 5 Precipitation process in HA synthesis.

BCP Synthesis

The solution of BCP in this research was prepared by a precipitation method. A hundred milliliters of 0.67 M $(NH_4)_2HPO_4$ solution was then be added dropwise to the 100 mL of 1 M CaO solution at the rate of 7 ml/min. Previously, CaO materials was prepared from chicken eggshell that calcined at 1000°C for 5 hours. The reaction was carried out at 300 rpm stirring. A white precipitate was obtained at the end of the reaction. The precipitate was heated hydrothermally at 300°C for 8 hours while stirring at 300 rpm. Then, the precipitate was be aged for 12 hours without stirring until it cooled down to room temperature. The solution was then be filtered under vacuum. The filtered cake was then being dried in the furnace at 110°C during 5 hours. The dried powder was then be sintered at 1000°C during 6 hours.

Figure 6 Hydrothermal processes in BCP synthesis.

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Preparation for In Vitro Analysis

BCP and HA need to be sterilized beforehand. Two milligram of BCP and HA powder was being put in each glass bottle that was being sterilized by gamma radiation with 25 kGy dozes.

Cells Culture

Culture medium was prepared in basic medium: DMEM supplemented with 10% FBS, penicillin streptomycin and fungizones. All basic medium was then melted beforehand inside 37°C-water bath within 15 minute. Osteoblast cell was be taken from liquid nitrogen storage (-198°C). The cells was then melted inside 37°C-water bath before being incubated for 24 hours at 37°C. The osteoblast cell wells washed with PBS before added by 1 mL-trypsin EDTA in order to release the attachment of cells from the bottom of the well. It was then be incubated again for 10 min (37°C) before replaced to the 15 mL-tube and added by basic medium. The 15 mL-tube was then centrifuge at 2000 rpm for 10 min (24°C) in order to concentrate the cell become a small pellet. Its supernatant needs to be removed and added by 5 mL of basic medium before homogenizing the pellet cell by several times pipetting in order to get cell solution.

Cells Concentration Counting

80 μL-cells solution, 10 μL-FBS and 10 μL-trypan blue was then mixed in the 1.5 mL-eppendorf tube. Ten micro liters of solution in eppendorf tube was then dropped to the hemocytometer glass board. The cells counting were done by counting the cells on hemocytometer glass under optical microscope with 40 times-magnification. It has the separation grid to counting the cells as shown Figure 7. A, B, C, D, and E is the result of cells counting manually under optical microscope. The cells concentration was being calculated using Equation 1.

Cell suspension was prepared with a concentration of 2×10^5 cells ml^{-1} and seeded into 96 well-plates. HA and BCP powder was being poured to well then be incubated at 37°C in an atmosphere containing 5% of CO_2 for 1, 2, and 3 days on each sample in triplicate as scheme on Figure 8.

$$\text{cell concentration} = \frac{A+B+C+D+E}{5} \times 10^4 \times 10 \times 5 \qquad (1)$$

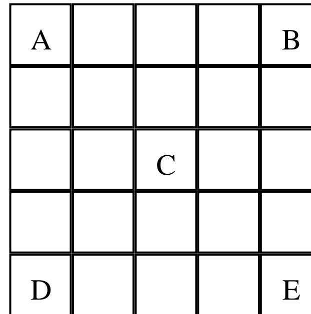

Figure 7 Scheme of grid on hemocytometer glass board

A is HA sample poured to the cells, B is BCP sample poured to the cells, C is the cells only and D is a blank that only contains basic medium (Figure 8). MTT test was performed to determine the cytotoxicity of BCP and HA. It was be calculated using the Equation 2.

$$\text{cell viability (\%)} = \frac{\text{sample absorbance value}}{\text{control absorbance value}} \times 100\% \qquad (2)$$

If the percentage of cell viability above 100 %, the materials exposed to the cell would be categorized as nontoxic.

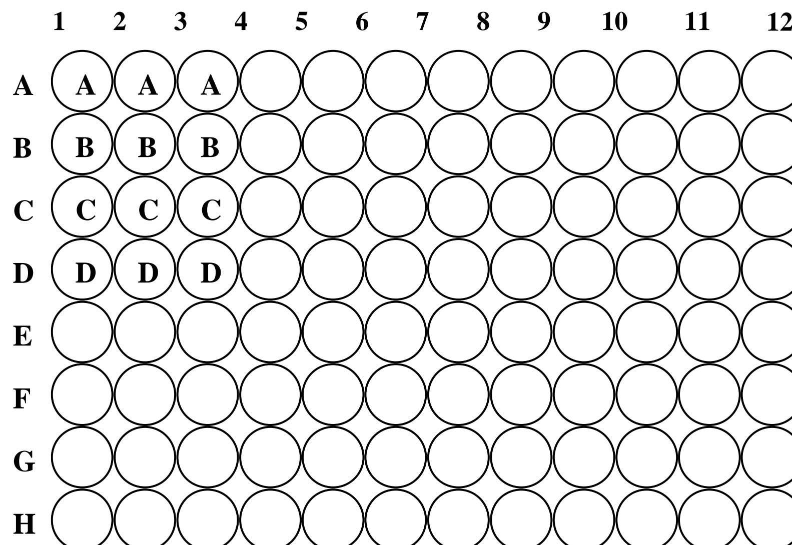

Figure 8 Scheme of 96-well plates for MTT analysis.

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Sample preparation for SEM characterization

The surfaces and biocompatibility were examined by SEM. For this purpose, after each culturing period, samples was being removed from culture, washed in PBS, fixed in 2.5% glutaraldehyde, rinsed two times with PBS and dehydrated in series of ethanol concentrations. The samples were then are dried at room temperature and sputter coated with gold before observation under the SEM.

Sample characterization

MTT analysis

The absorbance of cells was being analysis by visible spectrophotometer at wavelength 655 nm as shown on Figure 9. The output of absorbance measurement is performed in optical density (OD).

Scanning Electron Microscopy (SEM) characterization

Sample surfaces were being examined using a Scanning Electron Microscope after immersion in vitro (Figure 10a). Sample need to be coated by gold-palladium (80% of Au and 20% of Pd) beforehand. Coating process is using Ion Sputter JFC-1100 machine (Figure 10b). The magnification was being performed in 5000, 10000, 20000, and 40000 times- magnification.

Figure 9 Visible spectrophotometer for absorbance analysis.

(a) (b)

Figure 10 (a) Scanning electron microscope and (b) Ion Sputter machine.

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