Academic Sciences Asian Journal of Pharmaceutical and Clinical Research
Vol 5, Suppl 4, 2012 ISSN - 0974-2441

Research Article

DEVELOPMENT OF GROWTH PROTEIN DELIVERY SYSTEM IN BONE IMPLANT BASED BIONANOCOMPOSITE

RACHMAT MAULUDIN*, TRI SUCIATI, HANA PRATIWI

School of Pharmacy, Institut Teknologi Bandung , Bandung, West Java, Indonesia; Email: rachmat@fa.itb.ac.id

Received: 17 August 2012, Revised and Accepted: 18 September 2012

ABSTRACT

Currently tissue engineering therapy has been developed. In this therapy, bone formation cells were induced by biodegradable matrix (scaffold) and added by bone growth factor. Chitosan as matrix was chosen because of excellent biocompatibility, biodegradability and osteoconductive properties. Unfortunately chitosan has low mechanical strength. Chitosan was combined with hydroxyapatite (HA) to improve mechanical strength. Commercial hydroxyapatite was incorporated into chitosan matrix. The HA dispersion in matrix was extruted using needle size 27 gauge into NaOH solution until microspheres obtained. The microspheres were characterized using SEM and XRD. Dried microspheres were flushed by acetic acid and followed by pouring slurry into mold. Obtained scaffold was evaluated including to density, porosity and mechanical strength.

Redispersion commercial hydroxyapatite had particle size average of 472 nm. PXRD analyses revealed that all bionanocomposite has hydroxyapatite. The analyses were characterized by peak at 2θ of 25°. Morphology of the microspheres was analyzed by SEM and showed microsphere formed relative spherical but its surface was rough. Early analyses of protein entrapment showed that 27% protein was successfully loading scaffold. Density evaluation showed a tendency of increasing density by increasing hydroxyapatite content. Porosity was increase due to increasing of microsphere size of scaffold. An optimum mechanical strength was obtained in combination chitosan : hydroxyapatite = 80 : 20 with mechanical strength of 24.47 ± 0.45 Mpa. Increasing hydroxyapatite reduced mechanical strength due to hydroxyapatite can inhibit chitosan sintering process.

Keywords: Bionanocomposite, chitosan, hydroxyapatite, microspheres, mechanical strength, scaffold.

INTRODUCTION

Recently, bone fracture treatment is important issue in medicine field. Therefore, in the last two decade a new method of bone fracture treatment was developed using tissue engineering technology. This technique used natural processes of bone forming and induced bone forming cells into biodegradable matrix (scaffold) and continuous by adding bone growth factor.

There are many polymers which are normally used as scaffold material for instans: *Poly(ethylene glycol)*-PEG and *Poly(butylene terephthalate)*-PBT [1], *Poly(L-lactic acid)*-PLLA [2], *Poly(D,L-lactic-co-glycolic acid)*-PLGA [3, 4]. Unfortunately, those materials are synthetic polymers which are expensive and questionable regarding issue biodegradable in a body.

In addition chitosan is a natural biocompatible polymer which can be properly degraded in a body and has osteokonductive property.

Chitosan is considered as one of the most attractive natural biopolymermatrices for bone tissue engineering owing to its structural similarity to the glucoseaminoglycan found in bone. Chitosan, which can be degraded by enzymes in the human body and the degradation product is nontoxic, has been shown to promote growth and mineral rich matrix deposition in vitro and in vivo [5, 6].

Unfortunately chitosan has low mechanical strength. To overcome its restriction, chitosan microsphere was combined with hydroxyapatite (HA) in order to improve mechanical strength and get similar characteristic to natural bone tissue [7].

Hydroxyapatite (Ca10(PO4)6(OH)2; HAp) is the major inorganic component of natural bone and has been used as an orthopaedic and dental material, a column packing material for affinity chromatography to separate various proteins, and in industrial catalysts. Nano-HAp (nHAp) has been applied widely in medical field as a bone repair material because of its excellent bioactive and biocompatibility properties. HAp is known for its biocompatible, bioactive (i.e. ability of forming a direct chemical bond with surrounding tissues), osteoconductive, non-toxic, non-inflammatory, non-immunogenic properties. Thus, HAp is one of the ideal materials for bone substitutions due to the nature of its biocompatibility and mechanical strength. Other calcium phosphate apatites including sintered HAp have been widely used for repair and replacement of damaged or traumatized bone tissues.

Hydroxyapatite is main component of bone and known as *filler* in the *scaffold* because of osteoconductive property [8] dan biokompatibel [9].

Table 1: Bone composition [10]

Component	Amount
Hydroxyyapatite	69%
Organic matrix	22%
Collagen	90-96% of organic matrix
Others	4-10% of organic matrix
Water	9%

Scaffold is prepared by unification of the composite microspheres of chitosan/Hydroxyapatite. Microspheric *scaffold* has benefit in comparison with block *macroporous* namely it can used for filling into bone fracture with irreguler form and complex [11].

MATERIAL AND METHODS

Material

The material were used including to chitosan**,** hydroxyapatite (Sigma Aldrich), $Ca(NO_3).4H_2O$ dan $(NH_4)_2HPO_4$ (Merck) , CMC-Na, NH_4OH, Acetic acid, NaOH, bovine serum albumin (BSA) (Sigma Aldrich), QuantiPro™ Bicinchoninic acid Assay Kit (QPBCA kit, Sigma Aldrich),

Method

Hydroxyapatite synthesis

Hydroxyapatite was prepared using wet presipitation method. $Ca(NO_3).4H_2O$ dan $(NH_4)_2HPO_4$ were used as materials for Hydroxyapatite**.** For eassier understanding, detailed synthesis can be seen on Fig. 1.

Re-dispersion Hydroxyapatite

Hydroxyapatite was redispersed in CMC-Na 0.35% w/w solution with adjusted until pH of 10 and continoued by sonication in period of 2 minutes. Hydroxyapatite would be completely redispersed and continued by particle size analyzes using photon correlation spectroscopy (PCS).

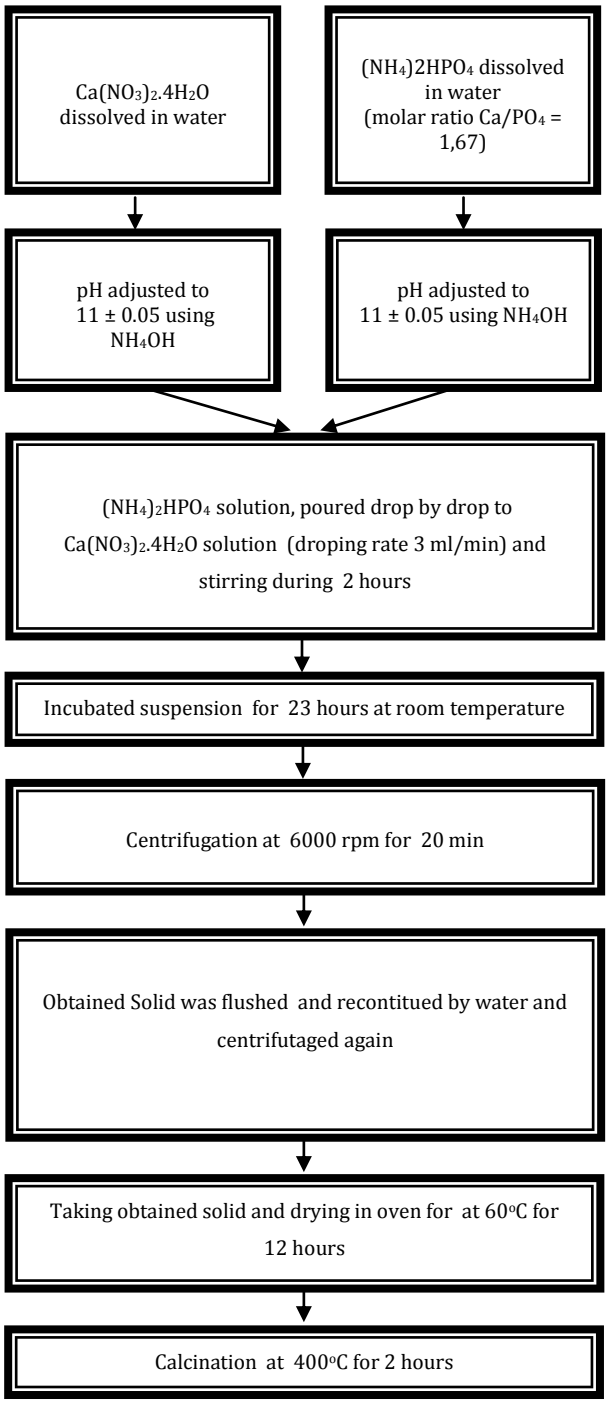

Figure 1: Hydroxyapatite synthesis flow chart

Microsphere composite forming

Chitosan as much as 1.2 gram was dissolved in 18 mL acetic acid (2%) using *stirrer* at rate of 200 rpm in period of 15 min. In addition, hydroxyapatite nanosuspension was added gentley to chitosan matrix. Rate of stirring was then increased until 300 rpm for 10 min. The rest of nanosuspension was added again to matrix and further stirred at 500 rpm for 15 min. Continued by adjusting pH to 5.5 ± 0.05 and volume to 40 mL. Stirring was continous again for 10 min to make sure that chitosan was dissolved already. After *degassing* process for 5 min, composite then extruded using 27 *gauge needle* into NaOH 5% solution and in same time stirring persistent continously using magnetic stirrer to form *crosslinking* in period of 1.5 hours. In the end microsphreres were opbtained. Finally microsphreres were washed by water, filtrated, and drying in incubator for over night.

Microsphreres characterization

Microsphreres were analyzed by PXRD and particles size distribution was determined by sieving at amplitudo 50 for 15 min. PXRD (Philips EXPERT PRO) was employed to identify and composite crystalinity characterization. PXRD studies were performed on the samples by exposing them to CuK_α radiation (40 kV, 25 mA) and scanned from 0.06° to 40°, 2θ at a step size of 0.04° and step time of 0.5 s.

In early investigation, bovine serum albumin (BSA) was used as protein model. Entrapped protein (BSA) in chitosan microsphere was determined using a QuantiPro™ Bicinchoninic acid Assay Kit (QPBCA kit, Sigma Aldrich).

***Scaffold* forming**

Obtained microsphreres were flushed with 5% acetic acid and pressed. Furthermore microsphreres were dried in an incubator for 24 hours. Resulted scaffold can be seen on Fig. 10.

***Scaffold* characterization**

Scaffold was analyzed regarding to density, porosity, and mechanic strength. Density was calculated with devided *scaffold* weight over its volume using caliper. Meanwhile porosity was determined using *liquid displacement method*. *Scaffold* was soaked in ethanol and placed in *orbital shaker* at room temperature and velocity of 100 rpm for 1 night. Please check to make sure that ethanol go inside matrix. Sign at early ethanol volume awal for V_1, *scaffold* and ethanol volume as V_2, and the rest of ethanol volume as V_3, then porosity can calculated through this equation:

$$porosity(\%) = \frac{V1 - V3}{V2 - V3} x100$$

Mechnical strength of scaffold was tested at LIPI Bandung with sample size as big as 1x1x2 cm. The unit for Mechnical strength is stated by MPa.

In addition morphology of each microsphere sample was observed using scanning electron microscope (SEM) apparatus. The material were placed onto carbon plates and coated under an argon atmosphere with gold to a thickness of 5nm. The samples were then observed with a scanning electron microscopy (JEOL JSM, tipe 6360LA) using secondary electron imaging at 10 kV.

RESULTS AND DISCUSSION

Hydroxyapatite synthesis

XRD data at figure 3 revealed that obtained solid as synthesis product consist of two phase namely hydroxyapatite and monetit. The two phase of solid can be characterized by spesific peaks at 2θ as finger print of the two phase of solid. Spesific peaks of Hydroxyapatite can be seen clearly at 2θ of 3 and 5°. Meanwile Spesific peaks of monetit can be seen clearly at 2θ of only 3°.

Figure 2: Left to right: hydroxyapatite preparation; result (A = hydroxyapatite synthesis, B = comersial hydroxyapatite)

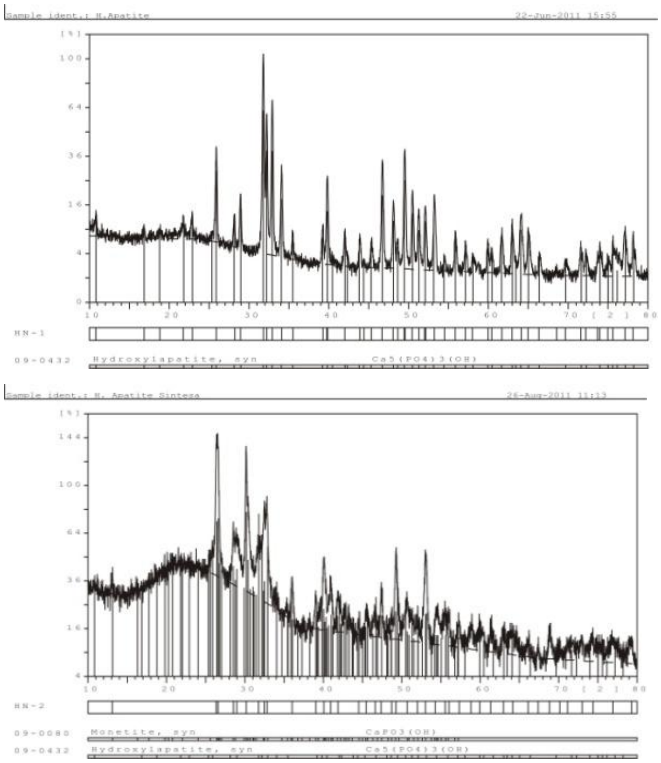

Figure 3: Top to down: X ray diffractogram of comersial hydroxyapatite and X ray diffractogram of synthesis hydroxyapatite

Presence another compound during hydroxyapatite production due to variation of synthesis process occurred. Variation of pH during precipitation and temperature variation during maturation process could produce another solid phase [12].

Re-dispersion Hydroxyapatite Nanoparticles

Before incorporation into chitosan matrix, de-agglomeration and hydroxyapatite re-dispersion were completed using sonication in CMC-Na solution. Ultrasonic wave could give forces to break interparticles bond at agglomerate. Addition CMC-Na solution can provide electrosteric barrier which polymer was adsorbed onto surface of ceramic (hydroxyapatite).

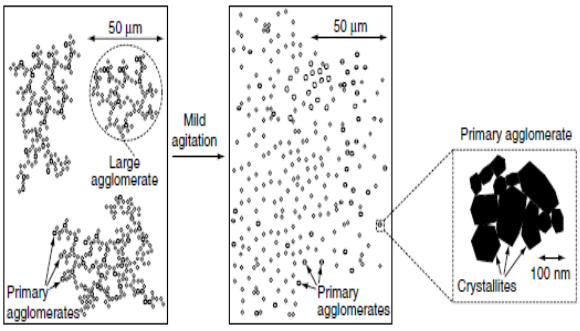

Figure 4: Hierarchies structure of ceramic powder [13]

Hydroxyapatite could be distributed in solution as single particle. Hopefully de-agglomeration and re-dispersion could break up the agglomerate and resulted prevalent distribution of hydroxyapatite in chitosan matrix.

Figure 5 showed a decreasing particle size average of hydroxyapatite before and after sonication. Particle size of comersial hydroxiapataite can be reduced from 4155.13 ± 141.71 nm to 472 ± 16.44 nm. Meanwhile particle size average of synthezed hydroxyapatite was decreased to 626.67 ± 58.76 nm from the original of 2988.13 ± 96.13 nm.

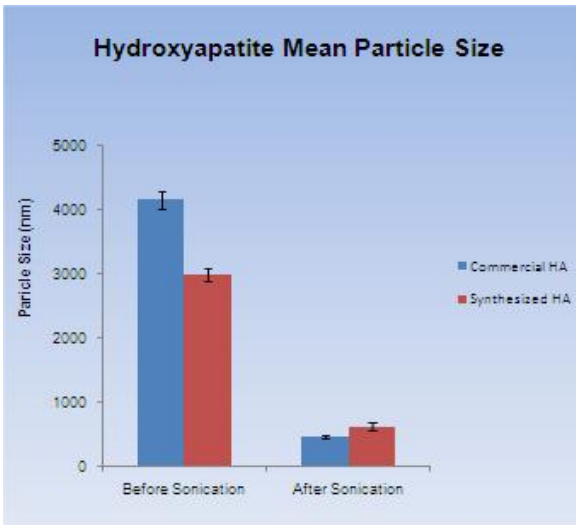

Figure 5: Graph of hydroxyapatite particle size average, after and before sonicasion.

Decreasing of particles size average was also accompanied with decreasing *Polydispersity Index (PI)* value concurrently. *Polydispersity Index* is a parameter to clarify particle size distribution. The smaller of *Polydispersity Index (PI)* value is more uniform of the particle size distribution. A monodisperse system has *Polydispersity Index* value of 0.01 and a wide particle size distribution is normally signed by *Polydispersity Index* value of more than 0.5.

The acceptation of *Polydispersity Index* value is 0.5, it is mean, the particle size distribution is relative narrow.

The PI value of comersial hydroxyapataite before sonication was 1.055 and decrease to 0.269 after sonication. Meanwhile The PI value of synthezed hydroxyapatite decreased to 0.264 from the original of 0.636. In addition, zeta potential value of comersial hydroxiapataite and synthezed hydroxyapatite is -33.10 ± 2.90 mV and -25.77 ± 2.62 mV respectively.

The differences of zeta potential value of comersial hydroxyapatite and synthezed hydroxyapatite depend on amount of absorbed CMC-Na onto surface of particles. CMC-Na is anionic polymer which is adsorbed by interaction with calcium ion onto surface of hydroxyapatite. Otherwise it can be adsorbed by hydrogen bond forming.

Microspheres forming and characterization

In the next image, it can be seen a microscopic picture of chitosan/ hydroxyapatite before extrusion. The figures reveal distribution of the particles which cannot be seen directly in the mixture. By the way at microscopic image can be seen only few of agglomerate particles.

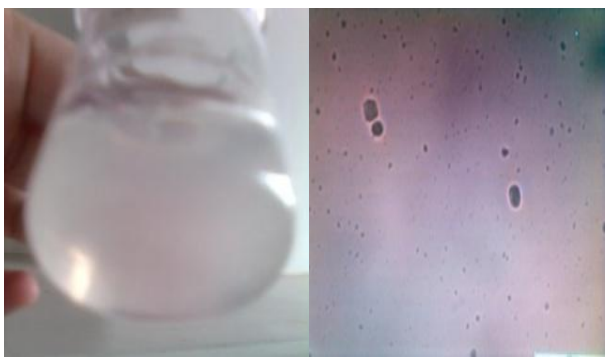

Figure 6: Left to right: visual image and microscopic image of chitosan-hydroxyapatite with magnification of 400x (75:1)

After extrusion of the mixture, microsphere can be observed by light microscope, as below image:

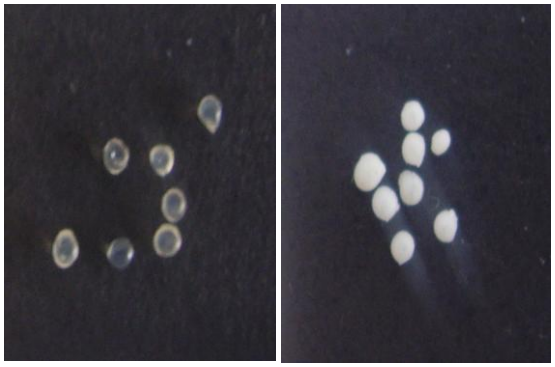

Figure 7: Left to right: chitosan microsphere; microsphere composite of chitosan /hidroxyapatite

At the left image, microsphere was transparent; meanwhile hydroxyapatite loaded-microsphere was white indicating microsphere contained hydroxyapatite.

XRD investigation

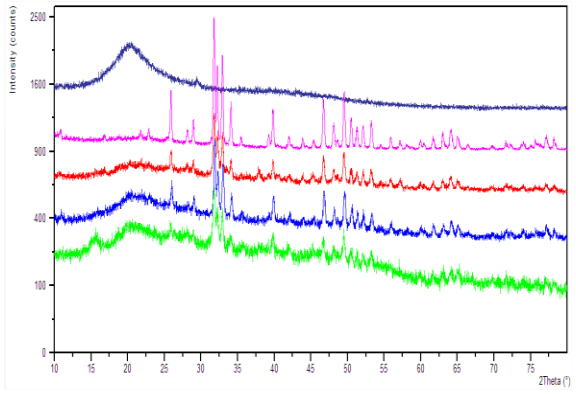

Figure 8: Top to down : chitosan, hydroxyapatite, composite with ratio chitosan : hydroxyapatite = 70:30, 80:20, dan 90:10

The microspheres were characterized by PXRD to determine crystalinity of hydroxyapatite loaded- microsphere. At diffractogram revealed that all microsphere consist of hydroxiapataite have peaks at 2θ around 25° and 32° which is the finger print of hydroxyapatite.

Particle size distribution of microspheres

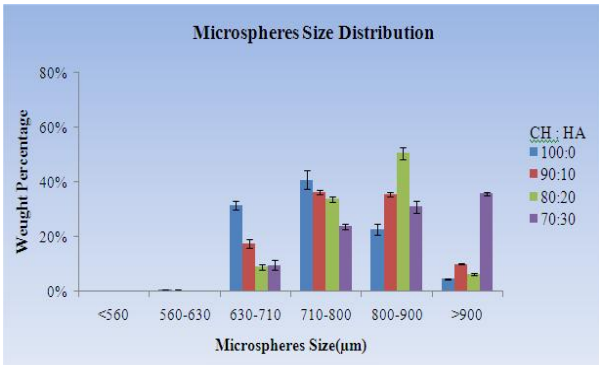

Figure 9: Particle size distribution of microspheres

Figure 9 revealed that the higher hydroxyapatite content in microsphere is the larger also particle size of microsphere. The size of microsphere is correlation with pore size of obtained *scaffold.* The bigger size of particle is also the bigger of a pore size. The pore size can provide a facility for bone tissue growth if the pore size more than 100µm [14].

Forming and characterization of *Scaffold*

At figure 10 can be seen prototype of *Scaffold* with various ratio of chitosan/hydroxyapatite. Higher content of hydroxyapatite in the Scaffold is larger also the pore size. According to Kawachi, larger of the pore size can give a facilitation to osteoblast for emergent.

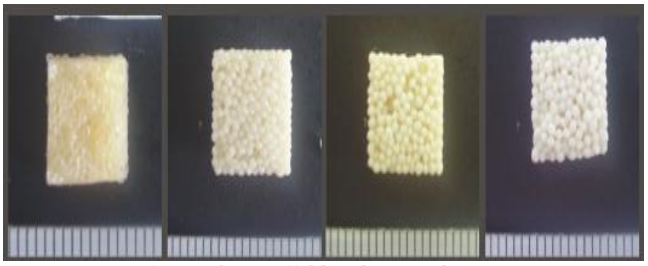

Figure 10: Left to right: *scaffold* with ratio chitosan to hydroxyapatite of 100:0, 90:10, 80:20, and 70:30

Density and Porosity

Table 2: Density and Porosity of Scaffold

CH:HA (%)	Density (g/cm³)	Porosity (%)
100 : 0	1.016 ± 0.023	53.95 ± 3.89
90 : 10	1.103 ± 0.038	72.74 ± 4.04
80 : 20	1.116 ± 0.026	75.1 ± 5.22
70 : 30	1.063 ± 0.022	81.90 ± 6.31

From the data above it known that the density tends to increase with increasing hydroxyapatite content inside of microspheres. In the same case with the porosity, increasing in porosity would be followed by increasing in the size of the scaffold.

Porosity and pore size are one of a most significant parameter in Scaffold forming. The volume of bone consists of about 30% of tissue and 70% of pore. Therefore, created *scaffold* must have a pore volume at least 30% for posiblelity bone regeneration and vascularitation occur [15]. All obtained scaffold met the requirement of porosity.

Mechanical Strength

Table 3: Mechanical strength

CH : HA (%)	Mechanical strength (MPa)
100 :0	18.97 ± 2.49
90 :10	18.94 ± 6.07
80 : 20	24.47 ± 0.45
70 : 30	8.74 ± 3.31

The optimum compressive strength obtained at the composition ratio of chitosan: hydroxyapatite = 80:20. Increasing loaded content of hydroxyapatite would decrease the mechanical strength. The existence of hydroxyapatite in the microsphere can hinder chitosan sintering process.

Protein entrapment efficiency

At early investigation, 27.04% of BSA as protein model was entrapped into scaffold. Further investigation is required to get optimum process in order more protein can be entrapped in the scaffold.

SEM evaluation

The pictures below show that the microspheres were relatively spherical in shape. The roughness was increased with increasing hydroxyapatite content. The roughness of microsphere surface has an advantage because it can increase adhesion and proliferation of the osteoblast [16].

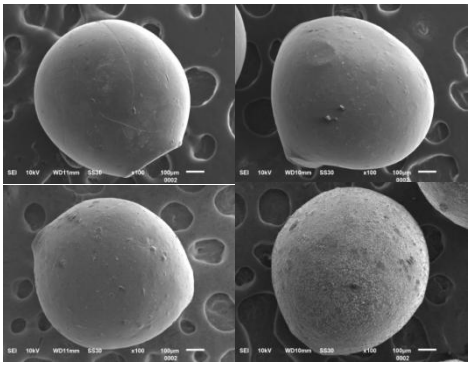

Figure 11: Left to right: Microsphere surface of Chitosan:Hydroxyapatite with ratio = 100:0, 90:10, 80:20, dan 70:30 at 100x magnification

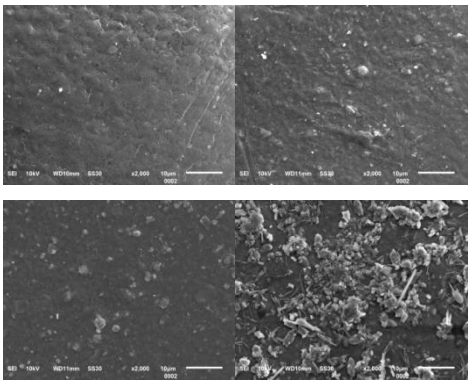

Figure 12: Left to right: Microsphere surface of Chitosan:Hydroxyapatite with ratio = 100:0, 90:10, 80:20, dan 70:30 at 200x magnification

CONCLUSION

The highest mechanical strength of the chitosan/hydroxyapatite bionanocomposite as much as 24.47 MPa was reached. It was achieved by a composite with a ratio of chitosan:hydroxyapatite = 80:20. Further optimization required to obtain the high protein entrapment efficiency.

ACKNOWLEDGMENT

This research was funded by DIPA in the 2011 budget for Research and Innovation Program KK ITB 2011.

REFERENCES

1. Liu Q, de Wijn JR, van Blitterswijk CA., 1998, "Composite biomaterials with chemical bonding between hydroxyapatite filler particles and PEG/PBT copolymer matrix", *J Biomed Mater Res,* Vol. 40, pp. 490–497
2. Wei G, Ma PX., 2004, "Structure and properties of nanohydroxyapatite/polymer composite scaffolds for bone tissue engineering", *Biomaterials,* Vol. 25, pp.4749–4757.
3. Kim SS, Ahn KM, Park MS, Lee JH, Choi CY, Kim BS. 2007, "A poly(lactide-co- lycolide)/hydroxyapatite composite scaffold with enhanced osteoconductivity", *J,Biomed Mater Res A,* Vol. 80, pp.206–215
4. Kim SS, Sun PM, Jeon O, Yong Choi C, Kim BS., 2006, "Poly(lactide-co-lycolide)/hydroxyapatite composite scaffolds for bone tissue engineering", *Biomaterials,* Vol. 27, pp. 1399–1409.
5. Katti K S, Katti D R and Dash R, 2008, "Synthesis and characterization of a novelchitosan/montmorillonite/hydroxyapatite nanocomposite for bone tissue engineering", *Biomed. Mater*, 3 (3), pp. 1-12
6. Katti K S, Turlapati P, Verma D, Gujjula P K and Katti D R, 2006, "Static and dynamic mechanical behavior of hydroxyapatite–polyacrylic acid composites under simulated body fluid", *Am. J. Biochem. Biotechnol,* pp.73-79
7. Boccaccini, A. R., and Blaker, J. J. , 2005, "Bioactive Composite Materials for Tissue Engineering Scaffolds", *Expert Rev. Med. Devices,* Vol.2, pp. 303–317
8. Lanza, Robert, et.al. 2007. *Principles of Tissue Engineering 3rd Ed*. Elsevier
9. Ducheyne, Paul. 1984. *Metal and Ceramic Biomaterials Vol.I Structure*. Florida: CRC Press Inc.
10. Yildirim, Oktay. 2004. Preparation and Characterization of Chitosan /Calcium Phosphate Based Composite Biomaterials. Department: Materials Science and Engineering, Izmir Institute of Technology, Turkey.
11. Wu, Chengtie,et.al. 2006. Bioactive Mesopore-Glass Microspheres with Controllable Protein-Delivery Properties by Biomimetic Surface Modification. Institute of Health & Biomedical Innovation, Queensland University of Technology, Australia.
12. Ravaglioli,A., et.al. 1992. *Bioceramics. Materials, Properties, Applications*. UK: Chapman & Hall.
13. Laarz, B. V. Zhmud and L. Bergstr¨om, 2000, *J. Am. Ceram. Soc.*, Vol. 83, pp. 2394–2700
14. Kawachi et.al. 2000, "Biocerâmicas: Tendênciase Perspectivas de uma Área Interdisciplinar", pp. 518-522
15. Borden, Mark, et.al. , 2002, "Tissue engineered microsphere-based matrices for bone repair: design and evaluation", *Biomaterials*, Vol.23, pp.551-559
16. Zan , Qingfeng .et.al. 2008. "Effect of surface roughness of chitosan-based microspheres on cell adhesion", *Applied Surface Science,* Vol. 255, pp. 401-403