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Biocomposite scaffold preparation from hydroxyapatite extracted from waste bovine bone

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Abstract: This study was conducted to fabricate scaffold from polylactic acid (PLA) and hydroxyapatite (HA) extracted from waste bovine bone for enhancing both mechanical and biocompatible properties. After pretreatment in dilute NaOH solution, the bone was calcined at 900°C for 6 h, ball milled and converted to HA. Factors that affect the formation of HA were investigated. Experimental results showed that HA particles with crystal size < 100 nm and 99% crystallinity could be obtained at 90°C, pH 11 and 35 mM H₃PO₄ solution followed by 4 h calcination at 900°C. By using non-solvent induced phase separation method, PLA scaffolds with pore size and surface area of 22.6 μm and 25.7 m²/g, respectively, containing different hydroxyapatite were successfully prepared. Tensile strength of scaffolds increased due to effective support by HA grafted collagen. PLA scaffolds containing HA were more degradable than PLA scaffolds and PLA scaffolds containing HA grafted collagen. Cell culture results showed that cell density increased significantly on porous scaffolds than that on the dense scaffolds. Moreover, cell density also increased significantly on the scaffold containing HA grafted collagen than that on the scaffold with pure HA.

Keywords: bovine bone; hydroxyapatite; phase separation scaffold; tissue engineering

1 Introduction

Tissue engineering method consists of harvesting cells from a patient, expanding them in culture, and seeding them into scaffolds which can later be implanted into the patient. The scaffolds known as artificial architecture for

biosynthetic extracellular matrix (ECM) are used to create a 3D environment in order to promote cellular attachment, migration, proliferation and differentiation. Therefore, finding materials to produce scaffolds that possess not only properties suitable for cell penetration, capillary ingrowth, and adequate solute transport but also good mechanical and biological properties is a very important work. Some synthetic polymers have been applied to create scaffolds such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA) [1], poly(lactic-co-glycolic acid) (PLGA) [2], poly(caprolactone) (PCL) [3], poly(propylene fumarate) (PPF), and poly(ethylene glycol) (PEG) [4]. However, mechanical properties of these synthetic polymers are very weak. Some fillers and reinforced materials such as calcium phosphate, ceramic and glass were mixed with polymers to enhance their mechanical properties [5–8]. However, fillers and reinforced materials usually have low biocompatibility. Therefore, it is necessary to find potential materials that can be applied in scaffold. Recently hydroxyapatite is considered as a good material to replace ceramic [9].

Hydroxyapatite [Ca₁₀(PO₄)₆(OH)₂] with molar ratio of Ca/P from 1.58 to 1.67 is well known as the mineral component of animal bones [10]. Hydroxyapatite (HA) was found to have good biocompatibility with cells [11]. HA can be synthesized by numerous techniques such as sol–gel, chemical precipitation, hydrothermal, hydrolysis, and microemulsion [12]. HA synthesized from chemical such as CaO, Ca(NO₃)₂, (NH₄)₂HPO₄ did not possess desired biological characteristics while these produced from natural materials such as egg shells or animal bones are very interesting since it is believed to be more biocompatible and biological safe [2–5]. However, high temperature treatment during extraction process may cause structure breaking or loss of certain components which will partly affect the biological activity of HA. In Vietnam, the yearly amount of bovine bones produced from food processing industry is huge. These bones are often used to produce low economic value animal feed. In this study, waste bovine bone was converted into HA and then applied to produce biocompatible scaffold by using phase separation method.

A simple nonsolvent induced phase separation method was widely used to prepare polymeric membrane

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[13,14]. This method is also considered as a potential method to create polymeric porous structure, especially scaffolds used in tissue engineering. In the nonsolvent method, polymer was dissolved in organic solvents such as tetrahydrofuran (THF), chloroform to form homogeneous polymer solution. The solution was then separated by adding nonsolvent such as water, acetone and ethanol to form porous structure. In this work, the nonsolvent induced phase separation method was applied to create porous scaffold from polylactic acid (PLA) and hydroxyapatite for tissue engineering.

2 Materials and methods

2.1 Materials

Bone of adult bovine (2-3 years old) was collected from a local slaughterhouse. The bone was first cooked at 100°C for 24 h to remove tissue, then dried at 80-100°C for 24 h and cut into small sizes of about 10 mm × 5 mm × 5 mm. Chemicals such as NaOH, H₃PO₄ and NH₃, 3-amino propyl triethoxysilane (APTES), glutaraldehyde (GA), and phosphate buffered saline (PBS) were supplied by Merck Vietnam for use in pretreatment and extraction of HA from bovine bone. Poly lactic acid (PLA) (CAS. 9051-89-2) was purchased from Nature Works. The solvents used in the preparation of scaffold such as chloroform (CHCl₃) and ethanol were supplied by Merck, Vietnam. Standard hydroxyapatite (≥ 90%) was supplied by Sigma- Aldrich, Vietnam.

2.2 Extraction of hydroxyapatite from bovine bone

After pretreatment with 0.1 M NaOH for 24 h, the pretreated bone was washed with water and acetone to remove fats and other impurities. The bovine bone was then calcined at 900°C for 6 h in a furnace (Nabertherm, LHT 1750°C) to remove all organic components in the bone. The calcined bovine bone was then grinded into powder with particle size of 5 to 10 μm by using high speed mill ball. The bovine powder (10 g) was dispersed in 100 mL distilled water. The mixture was then reacted with aqueous H₃PO₄ solution. The reacted mixture was cooled to room temperature and neutralized by NH₃ in 2 h. After reaction, the mixture was aged overnight before separating by filter. The sample was dried at 100°C before continuously calcining at high temperature to stabilize the structure of HA. Factors that

affected HA formation such as reaction temperature, reaction time, pH and concentration of H₃PO₄ were investigated. X-ray diffraction (XRD), scanning electron microscopy (SEM), and Fourier transform infrared spectroscopy (FTIR) were applied for material characterization.

2.3 Preparation of hydroxyapatite grafted collagen

HA extracted from bovine bone was dispersed (1 wt%) in a co-solvent of ethanol/water (1:2 v/v). The suspension was added sequentially APTES 2 wt% and NH₃ 6 wt% while stirred at 100 rpm in an environment of N₂ for 24 h. HA-APTES was collected and washed with distilled water 3 times. The HA-APTES was dispersed (1 wt%) into aqueous solution of 1% glutaraldehyde at room temperature and stirred at 100 rpm for 24 h. The mixture was filtered and washed by PBS solution to obtain the HA-APTES-GA particles. The particles were dispersed 1% in PBS solution containing 100 mg/mL collagen extracted from catfish skin. The mixture was then stirred at 100 rpm for 24 h at 4°C. HA grafted collagen obtained was washed three times with PBS solution.

2.4 Preparation of scaffold

Nonsolvent induced phase separation method was applied to prepared scaffold. Poly lactic acid (PLA) was dissolved in chloroform at room temperature for 24 h. After that the polymer solution was casted onto a teflon mold (5 mm × 60 mm × 100 mm). The teflon mold was then immersed in a coagulation bath containing nonsolvent (ethanol/water) and PLA porous scaffold was obtained after 24 h.

2.5 Cell culture

2.5.1 Cell seeding

Fibroblast L929 standard cell suspension containing 20,000 cells/mL was prepared by diluting the cell suspension with serum medium. One milliliter of the cell solution was distributed in 24-well plate containing samples and incubated at 37°C in 5% CO₂ atmosphere for 24 h. After a certain incubation time, the culture medium was removed and the unattached cells was washed by PBS solution. Cell density was characterized by both lactate dehydrogenase (LDH) assay and confocal laser scanning microscope.

2.5.2 Cell density by lactate dehydrogenase (LDH) assay

Cell density was determined by lactate dehydrogenase assay [15-18]. The attached cells were lysed with 100 μ L of 1% Triton X-100 in PBS. The reaction solution contained 0.3% NAD⁺, 0.27% diaphorase, 0.03% bovine serum albumin, 1.2% sucrose, 0.02% iodonitrotetrazolium, and 3.6% sodium lactate (all chemicals were purchased from Sigma). Optical density values were read at 490 nm by using enzyme-linked immunosorbent assay (ELISA, iMark™ Absorbance Reader, BioRad). A standard curve was obtained by plotting the measured optical density of a series of cell solutions verses known cell densities.

2.5.3 Immunofluorescence staining for microscopy observation

The L-929 fibroblasts were fixed on the scaffold surfaces by immersion in a solution of 4% paraformaldehyde in PBS, pH 7.4, followed by permeabilization with 0.1% Triton X-100 at room temperature. Non-specific binding of proteins and antibodies was prevented by incubation with 0.5% BSA solution in PBS overnight at 4°C. The cells were stained for actin filaments with 165 nM of Rhodamine-FITC (Fluka, 77418) for 20 min at room temperature. The nuclei were stained with DAPI (Sigma, CAS. 28718-90-3) at 1:1000 dilutions for 20 min at room temperature.

2.5.4 Confocal laser scanning microscope

Confocal laser scanning microscope (LSCM, Leica-SP2, Germany, 40× 0.75 dry, 60× 0.75 oil or 100× 1.4 oil objective) was used to image the surface morphology of stained cells. Fluorescence with excitation wavelength (Ex) = 364 nm and emission wavelength (Em) = 454 nm were used to image cells stained by DAPI; and fluorescence with Ex = 495 nm and Em = 520 nm were used to capture images of cells stained by Phalloidin-FITC.

2.5.5 Statistical analyses

Variations of the amount of adherent and proliferate cells were statistically analyzed by performing one-way analysis of variance (ANOVA), executed by Minitab statistical software®. Fisher's pairwise comparison test was applied to compare cell density on different samples for $p < 0.05$.

3 Results and discussion

3.1 Hydroxyapatite extraction from waste bovine bone

Bovine bone was pretreated to remove tissue, fats and other impurities. As shown in Figure 1, the color of bone was

Figure 1: Pictures of bovine bone treated by: (a) hot water at 100°C, 24 h; (b) 0.1 M NaOH solution, 48 h; (c) calcination at 900°C, 6 h; (d) ball milling for 30 min.

Table 1: Chemical composition of calcined bovine bone powder.

Composition	SiO ₂	Al ₂ O ₃	Fe ₂ O ₃	CaO	MgO	SO ₃	K ₂ O	Na ₂ O	Cl	P ₂ O ₅
Weight %	-	0.49	0.01	44.10	1.41	1.05	0.02	3.69	0.04	36.2

Figure 2: XRD diffraction of HA that was prepared in different concentrations of H₃PO₄: (a) 0 M, (b) 25 mM, (c) 30 mM, (d) 35 mM, and (e) Standard HA. Fixed condition: bovine powder/H₃PO₄ = 1 g/10 mL, reaction temperature 80°C, pH 11 and calcine temperature 900°C.

Table 2: Effect of reaction temperature on size and crystal degree of HA.

H ₂ SO ₄ concentration, mM	0	25	30	35
Size of crystal, nm	84.8	83.6	86.4	88.3
Degree of crystallinity, %	88.7	94.4	93.1	95.9

dark brown after cooking for 24 h at 100°C and it became brighter after treatment with NaOH solution. The bone was bright white and lost 45 wt% after calcined at 900°C.

Chemical composition of the powder of bovine bone was analyzed by X-ray fluorescence (XRF). The results show that calcium and phosphorus are the two

Figure 3: XRD diffraction of HA sample synthesized at (a) 60°C, (b) 70°C, (c) 80°C, (d) 90°C and (e) Standard HA. Fixed conditions: bovine powder/H₃PO₄ = 1:10 g/mL, H₃PO₄ (35 mM), pH 11 and calcine temperature 900°C.

main components in bovine bone powder. CaO and P₂O₅ accounts for 44 wt% and 36 wt% of bovine bone powder, respectively (Table 1).

3.1.1 Effect of concentration of H₃PO₄ on HA product

Results of XRD diffraction analysis of the calcined bovine bone show peaks of HA at 2θ (degree) at 10.8, 25.9, 31.7, 32.1, 34.1, 39.8, 46.7, 49.5, 50.5, 53.2; together with peaks of β-TCP and CaO at 2θ = 31.3, 33.67, 34.6, 35.85 and 23.4, respectively (Figure 2). It means that after calcining, crystal structure of HA, β-TCP and CaO still exist. Thus, in order to fully convert β-TCP and CaO that was created during calcination, H₃PO₄ with different concentrations was used to react with bovine bone powder. Results show that as concentration of H₃PO₄ increased, the peaks of β-TCP and CaO gradually disappeared and the intensity of peaks for HA increased. The peaks of β-TCP and CaO almost disappeared when using 35 mM H₃PO₄ and its characteristic

peaks is not very different with the peaks of standard HA (Figures 2d and 2e). Calculated base on the Scherrer equation, the size of HA crystal was 83, 85, 86 and 88 nm and degree of crystallinity was 88%, 93%, 94% and 96% corresponding to concentration of H₃PO₄ 0 mM, 25 mM, 30 mM and 35 mM, respectively (Table 2). Therefore, H₃PO₄ 35 mM is considered as a good concentration to convert bovine bone to HA.

3.1.2 Effects of reaction temperature on formation of HA

Reaction temperature was varied from 60°C to 90°C to investigate its effect on HA formation. From the XRD diffraction (Figure 3), HA samples synthesized at 60°C, 70°C and 80°C showed that besides the characteristic peaks of HA, there still existed a few characteristic peaks of β-TCP. At 90°C, only the characteristic peaks of HA appeared. This result shows that reaction rate increased with increasing reaction temperature and at 90°C high impurity HA was created. This agrees with the results of Luo [19]. Moreover, besides increasing intensity, the HA peaks also become sharper which means that crystallinity increased with increasing reaction temperature. As temperature was increased from 60°C to 90°C, the degree of crystallization of HA increased from 93.5% to 97.7%,

Table 3: Effect of reaction temperature on size and crystal degree of HA.

Reaction temperature, °C	60	70	80	90
Size of crystal, nm	80.3	86.5	88.3	95.7
Degree of crystallinity, %	93.5	94.8	95.9	97.7

Figure 4: XRD diffraction of HA sample synthesized at: (a) pH 9 (b) pH 10, (c) pH 11, (d) pH 12 and (e) Standard HA. Fixed conditions: bovine powder/H₃PO₄ = 1:10 g/mL, H₃PO₄ (35 mM), reaction temperature 90°C, calcine temperature 900°C.

accompanying with the increase of crystal size from 80.3 to 95.7 nm (Table 3). This result is consistent with the study of Wijesinghe et al. [20]. Therefore, 90°C is the suitable temperature to produce HA from bovine bone.

3.1.3 Effect of pH on formation of HA

HA was synthesized at different pH values to investigate pH effect on the morphology and structure of HA. The XRD diffraction in all samples showed the characteristic peaks of HA appear (Figure 4). However, at pH 9, pH 10 and pH 12, there were few characteristic peaks of β -TCP. This result is consistent with the previous study of Peipei Wang et al. [21]. HA synthesized at pH 11 showed the characteristic peaks of HA and reach the highest crystal level of 97.7% (Table 4). The size of the crystal tends to increase with increasing pH and this is consistent with the study of Liu et al. [22].

Table 4: Effect of reaction temperature on size and crystal degree of HA.

pH value	9	10	11	12
Size of crystal, nm	89.3	91.9	95.7	104.5
Degree of crystallinity, %	94.3	94.4	97.7	94.9

Table 5: Effect of calcine temperature on crystal size and crystal degree of HA.

Calcination temp., °C	700	800	900	1000
Size of crystal, nm	90.6	92.5	95.7	101.8
Degree of crystallinity, %	83.7	95.9	97.7	99.0

Figure 6: HA applied for grafting collagen was prepared under condition of calcine temperature 1000°C, bovine powder/ H_3PO_4 = 1:10 g/mL, H_3PO_4 , 0.35 M, reaction temperature 90°C and pH 11.

Figure 5: XRD diffraction of HA sample at: (a) 700°C, (b) 800°C, (c) 900°C, (d) 1000°C and (e) Standard HA. Fixed conditions: bovine powder/ H_3PO_4 = 1:10 g/mL, H_3PO_4 (35 mM), reaction temperature 90°C and pH 11.

3.1.4 Effect of calcination temperature on HA formation

Temperature was varied from 700°C to 1000°C to investigate its effect on the formation of HA. Results of XRD diffraction analysis showed that the characteristic

Figure 7: FTIR spectra of collagen grafted HA sample.

peaks of HA existed in all samples (Figure 5). The samples synthesized at 700°C and 800°C still showed small characteristic peaks of β -TCP phase and these peaks disappeared as temperature was increased to 900°C. At 900°C, only the characteristic peaks of HA appear and these peaks become narrower and sharper at 1000°C. Moreover, as temperature was increased from 700°C to 1000°C, crystallization degree increased from 83.7% to 99.0%, accompanying by the increase of crystal size from 90.6 nm to 101.8 nm (Table 5).

3.2 Preparation of hydroxyapatite grafted collagen

HA prepared under optimal conditions of calcine temperature 1000°C, bovine powder/ H_3PO_4 = 1:10 g/mL, H_3PO_4 (35 mM), reaction temperature 90°C and pH 11 was used for grafting collagen. SEM results (Figure 6) show that HA particles have asymmetric spherical form with size of about 1000 nm. The FTIR spectra (Figure 7) of the collagen grafted HA particles show the characteristic peak of HA

Figure 8: SEM images of scaffolds prepared in different HA contents: (a) 0%, (b) 5%, (c) 10%, (d) 15%, (e) 20%.

Figure 9: Tensile strength of scaffold containing HA and HA grafted collagen.

Figure 10: Degradation of scaffold in PBS solution.

Figure 11: LDH results for cell density on pristine (dense PLA scaffold), PLA-HA scaffold and PLA-HA-Col scaffold at different concentrations of HA particles.

–OH (3437 cm^{-1}), PO_4^{3-} (1042 ; 568 cm^{-1}) and characteristic peak of –CO– (1997 cm^{-1}) and –NH₂ (3572 cm^{-1}).

3.3 Preparation of biocompatible composite scaffold

Scaffolds in different concentrations of HA and HA grafted collagen were prepared by using nonsolvent induced

phase separation method. PLA solution (125 g/L) was prepared in chloroform at room temperature. The polymer solution was mixed with HA and casted onto a teflon mold ($5\text{ mm} \times 60\text{ mm} \times 100\text{ mm}$). The teflon mold was then immersed into a coagulation bath (25°C , volume ratio ethanol to water $96/4$; volume ratio solvent to nonsolvent $1/20$). PLA porous scaffolds with different HA contents were obtained after for 24 h. SEM images show porous

Figure 12: Confocal laser scanning microscopy images of L-929 fibroblasts on scaffolds, cultured for 24 h: (a) TPCS, (b) PLA dense scaffold without pore and (c) porous scaffold without HA. (d), (e) and (f) are the images from PLA scaffolds that contained HA ranging from 5%, 10% to 15% respectively and (g), (h) and (k) are the images from PLA scaffolds that contained HA grafted collagen ranging from 5%, 10% to 15%, respectively (scale bar = 50 µm).

structure and its average pore size is $22.6 \pm 4 \mu\text{m}$ (Figure 8). BET measurement resulted in a surface area of $25.7 \text{ m}^2/\text{g}$.

Mechanical property of scaffolds was evaluated by tensile strength measured on a Zwick/Roell BDO equipment – FB050TN. Tensile strengths were 88.5 MPa, 76.5 MPa, 62.3 MPa, 40.1 MPa, and 10.6 MPa for scaffold samples containing 0%, 5%, 10%, 15%, and 20% HA, respectively (Figure 9). The results mean that the interface of HA particles and PLA was not compatible leading to decrease in tensile strength when HA content was increased. Compatibility was significantly improved for collagen grafted HA. Tensile strength of scaffold increased with increasing HA grafted collagen from 5% to 10% and after that decreased from 15% to 20%.

3.4 Degradation of scaffold in PBS solution

Scaffold samples containing 10% particles were prepared with size of $1 \times 1 \text{ cm}^2$ and immersed in 10 mL PBS solution at 37°C . After 7 days, degradation of scaffolds was 0.45%, 0.5% and 0.75% for PLA, PLA-HA-Col and PLA-HA, respectively. The degradation of scaffold increased with time. After 28 days, degradation was 1.75%, 3.4% and 4.3% for PLA, PLA-HA-Col, and PLA-HA, respectively. The results show that all scaffolds could be degraded in PBS solution (Figure 10). The degradation is highest for the PLA scaffolds containing HA. However, after grafting with collagen the degradation is lower than PLA scaffold, meaning that the interaction between PLA and HA-Coll is better than that of PLA and HA.

3.5 Cell responses on scaffolds

Biocompatibility of scaffold was studied based on cell responses. PLA scaffolds containing different concentrations of HA particle and collagen grafted HA particle were evaluated by directly culturing L929 cell line on the samples and characterized by lactate dehydrogenase assay and confocal microscopy. Cell density is shown in Figure 11. Cell density increased significantly on porous scaffolds compared to that on dense scaffold. Cell density also increased slightly as HA content was increased from 0% to 10% and increased significantly as HA content reached 15%. Moreover, cell density also increased significantly on scaffold containing HA grafted collagen compared to that on scaffold with pure HA.

Confocal microscopy images show that fibroblasts attached on PLA dense and PLA scaffold without HA with limited number after 24 h cultivation (Figures 12a and 12b). It is similar to LDH results, confocal microscopy images also show that cell number increased approximately with increasing HA concentration in porous scaffold (Figures 12d-f), suggesting that cell proliferation was promoted by HA. Moreover, it was observed that higher cell density was found on surfaces containing HA grafted with collagen (Figures 12g-k).

4 Conclusion

An effective method was successfully applied to produce hydroxyapatite from waste bovine bone. It was possible to create HA particles with a crystal size < 100 nm with a crystallinity of 99% under conditions of H_3PO_4 35 mM, reaction temperature 90°C, pH 11, calcination at 900°C for 4 h. Biocompatible PLA scaffolds prepared by combining HA promoted cell growth effectively depending on HA content. Collagen extracted from catfish was successfully grafted on HA surface and it enhanced cell proliferation compared to pure HA.

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