Effect of Polyvinyl Alcohol (PVA) Blending and Gamma Irradiation on Compressive Strength of FHAp/FGel Composite as Candidate of Scaffold

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***Abstract*—Composite as candidate of scaffold have been synthesized from fish hydroxyapatite (FHAp) and fish gelatin (FGel) of Barramundi (*Lates calcarifer* Bloch) scale that aqua-cultured in Jakarta Bay, Indonesia. The effect of polyvinyl alcohol (PVA) blending was investigated for improvement of compressive strength of composites. Composites were synthesized by wet method at FGel/PVA percent mass ratio of 10/0, 7.5/2.5, 5.0/5.0, and 7.5/2.5 (w/w), respectively. Sodium citrate was used as dispersant to forming a paste-like suspension condition. Porous composites were obtained after freeze-drying of suspensions. Compressive strength and Young's modulus of scaffold were increased as increasing PVA content. FTIR spectra confirmed the molecular interaction among components of composite. SEM micrograph showed fibrous and ribbon structure over surface of FHAp/FGel composite that blended with PVA. Gamma irradiation lead to formation of micro- and mesopores in the wall surface of composite.**

Keyword- Fish hydroxyapatite, Fish gelatin, Polyvinyl alcohol, Composite, *Lates calcarifer* Bloch

I. INTRODUCTION

Composite synthesized from collagen and hydroxyapatite are known as a candidate of scaffold because their composition is similar to the extracellular matrix of natural bone [1-3]. More recently, gelatin has been used as replacement of collagen since it is less expensive and easier to obtain. Similar to collagen, gelatin also contains RGD (*Arg-Gly-Asp*) peptide sequences which enhance cell proliferation and differentiation [4]. Even gelatin based materials shows excellent cyto-compatibility, its long-term application is limited by its brittleness in dry state [5].

Possible alternative to improve the compressive strength of HAp/gel composite is by blending with synthetic. Low density polyethylene is the most studied synthetic polymer blended with natural polymer [5], but this polymer needs chemical treatments to become biocompatible [6]. Polyvinyl alcohol (PVA) has also gain attention for improvement of mechanical properties of proteins based polymer because it is a water soluble synthetic polymer with non-toxic, non-carcinogenic, and good biocompatibility properties [7]. Despite its synthetic character, this polymer was recognized recently as biodegradable [8]. PVA has been used in a number of biomedical application including contact lenses, cartilage implant, drug-delivery matrices, burn dressing, and artificial organs [9].

Improvement on the solubility and mechanical properties of HAp/gel scaffold may also be achieve by induce chemical crosslinking reaction through covalent bonds. Numerous data on chemical crosslinking of gelatin can be found in literature by involving chemical cross-linker [10], photo-initiator, enzyme [11], or radiation crosslinking [12, 13]. Reference literature states that the use of gamma radiation for biomedical materials is preferable as it allows eliminating residual initiators/cross-linker and subsequently sterilized the product if irradiated at 25 kGy or higher. PVA solution may also cross-linked by gamma irradiation [12, 13].

Several studies about improvement on solubility and mechanical HAp/gel scaffold have been conducted, but most of these studies have been focused on mammalian gelatin and synthetic hydroxyapatite. Due to various reasons, alternative sources such as fisheries waste was proposed as sources of both materials. Biological hydroxyapatite from bio-waste is considered as a biologically safe and a potentially lucrative process, especially given the growing demand for hydroxyapatite [14]. While, the use of fish gelatin have been driven by the fear of zoonosis such as bovine spongiform encephalopathy and swine flu, and also due to religious consideration of halal products in Muslim community [15]. Recently, waste from various fish species such as from barramundi [16, 17] and sardines [18] has been extracted for fish hydroxyapatite (FHAp), as well as barramundi [19] and baltic code [20] for fish gelatin (FGel).

In this study, FHAp/FGel composites were synthesized from FHAp and FGel derived from Barramundi (*Lates calcarifer* Bloch, local name *ikan kakap putih*) fish that aqua-cultured in Jakarta Bay, Indonesia. Barramundi is one of important aqua-cultured fish species in Indonesia with total production of 3,609 tons in 2012 with annual grow rate of 18% during 2009-2012 [21]. The use of fishery waste from aquaculture production is a rational strategy for quality control of the raw material [15]. Fisheries waste that used was in form of scale because fish scale is considered worthless, impracticable, and dismiss as a waster [22]. Compressive strength of FHAp/FGel based scaffolds were measured. Molecular interaction among materials in composite were characterized by Fourier Transform Infrared (FTIR) spectroscopy. Surface morphology of composites were observed using scanning electron microscope (SEM).

II. MATERIALS AND METHODS

A. Materials

FHAp and FGel derived from Baramundi (*Lates calcarifer* Bloch) scale were obtained from Centre for Application of Isotopes and Radiation, National Nuclear Energy Agency (CAIR-NNEA), Republic of Indonesia. The FGel is a type A gelatin in form of yellowish transparent film with molecular weight of 50-75 kDa. The FHAp is in white powder form, 118.9 nm in average particle size, hexagonal crystal system, and 1.72 in Ca/P ratio. PVA (Merck, MW 72,000 g/mol, 98 % degree of de-acetylation) and tri-sodium citrate di-hydrate (Merck, MW = 210.14) were of analytical grade.

B. Preparation of Irradiated FHAp/FGel based Composite

A series of 200 ml FGel/PVA solution were prepared by blending 10 % (w/v) FGel solution with 10 % (w/v) PVA solution at percent of mass ratio at 10/0, 7.5/2.5, 5.0/5.0, and 2.5/7.5 (w/w), respectively. After homogenously stirred, a 20 g of FHAp powder was added to each of FGel/PVA solution to obtaining 10 % (w/v) FHAp suspension. Then, FHAp/FGel based suspensions were stirred for 30 minutes to at room temperature followed by high-shear blending at 10,000 rpm for 2 x 5 minutes in the presence of 2.86 g sodium citrate. Sodium citrate used as a dispersant to reduce the sedimentation of HAp in suspension [23]. Each of 200 ml FHAp/FGel-PVA suspension was stirred for 3-5 hours at room temperature to forming a paste-state suspension.

A 200 ml FHAp/FGel-PVA suspension was distributed to four of 6 x 6 x 1.5 cm^3 polypropylene bags. Each bag was gamma irradiated at dose of 0, 15, 30, and 45 kGy, respectively, at gamma irradiator facility of Iradiator Karet Alam (IRKA), CAIR-NNEA. Radioactive source in irradiator facilities was Cobalt-60 source with activity of 89.396 kCi in December 2014. Sample holder was located at 0.5 m from Co-60 source which given dose rate of 4.303 kGy/hour. Irradiated suspensions were stored in refrigerator at -20°C for 24 hour. Frozen samples were aseptically cut into dice shape with height of 3-5 mm, put in petri disc, then freeze once more in refrigerator at -80°C for next 24 h. After two steps of freezing condition, the samples were put into freeze-dryer (Beta I, Christ) at chamber temperature of -80 $^{\circ}$C, chamber pressure of 10^{-3} mbar for 3 days.

C. Measurement of Compressive Strength

Compressive test of composites were measured using universal testing machine (model 5944 Single column Table Top System, Instron) with maximum stress at 2 kN at ambient temperature. Compressive test was conducted at dry state according to standard ASTM F451-08 with modification. Cube shaped sample of about 6 mm x 6 mm x 6 mm were tested at cross-head speed of 1.0 mm/min and until 10% deformation in specimen height. The compressive strength at yield (σ_s) and Young's modulus (E) were determined based on the stress-strain curve from instrument record.

D. FTIR Characterization

The FTIR instrument (IR-Prestige21 model 800 series, Shimadzu) was used to characterize the presence of specific chemical groups in scaffolds. FTIR spectra were obtained over the range of 4000-400 cm^{-1}. Sample was milled and mixed with dried KBr powder placed in a sampling cup. Sample was scanned 20 times at 2 cm^{-1} resolution with subtraction of the KBr background. FTIR spectra are also obtained for fish HA powder, fish gelatin film, and PVA for comparison with samples.

E. FTIR Characterization

The surface morphology of irradiated FHAp/FGel based scaffold was studied using a scanning electron microscope (EVO MA10, Zeiss) at accelerating voltage of 5.0 kV. SEM specimens were sputter-coated with carbon.

III. RESULTS AND DISCUSSION

A. Text Font of Entire Document

The FHAp/FGel composites have been synthesized from Barramundi (*Lates calcarifer* Bloch) scale derived FHAp and FGel. Effect of PVA blending and gamma irradiation on their compressive strength at yield (σ) and Young's modulus (*E*) were shown in Fig. 1 and Fig. 2, respectively.

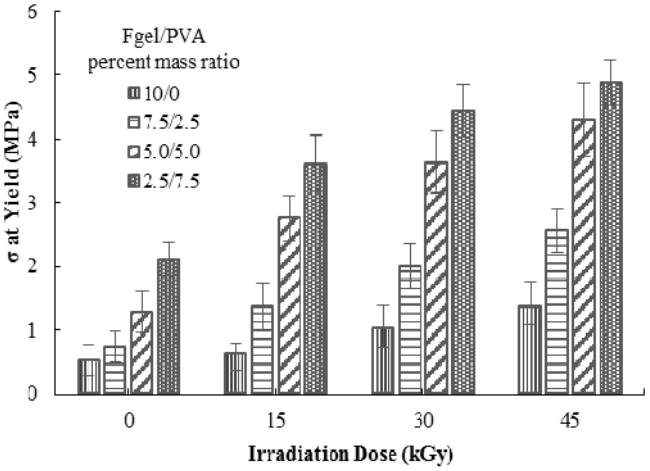

Fig. 1 Compressive strength at yield (σ_s) of irradiated FHAp/FGel based composite at varied FGel/PVA mass ratio

Compressive strength at yield of scaffolds were significantly increase as increasing content (*two-way anova*, $F_{(3,64)} = 242.999$, $p = .000$) as well as increasing irradiation dose (*two-way anova*, $F_{(3,64)} = 122.153$, $p = .000$), as shown in Fig. 1. Controlled FHAp/FGel scaffold showed lowest value at 0.524±0.238 MPa, while composites treated with combination of highest PVA content and highest irradiation dose showed highest value at 4.885±0.362 MPa. However, this is not the true value related to modification of sample shaped. The value of compressive strength at yield in this study is need to be subtracted by 1.25 as a conversion factor of compressive strength of cube shaped sample to standard cylindrical shaped sample [24]. Significant interaction (*two-way anova*, $F_{(9,64)} = 7.407$, $p = .000$) was occurred between both treatments. Regression analysis revealed that the slope of curves *irradiation dose vs compressive strength* were lower as increasing PVA content i.e. at 0.0195 (R^2=0.95), 0.0407 (R^2=0.99), 0.0661 (R^2=097), and 0.0612 (R^2=0.94) for FGel/PVA percent mass ratio of 10/0, 7.5/2.5, 5.0/5.0, and 2.5/7.5 (w/w), respectively.

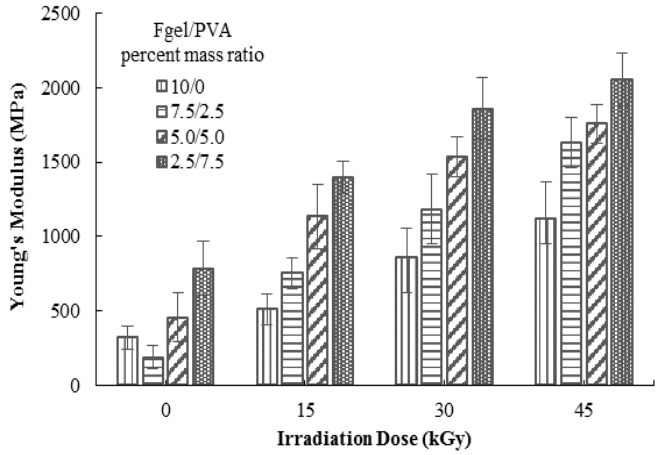

Fig. 2 Young's modulus (*E*) of irradiated FHAp/FGel based composite at varied FGel/PVA mass ratio

Young's modulus of scaffolds were also significantly affected by PVA blending (*two-way anova, $F_{(3,64)}$ = 90.670, p = .000*) and gamma irradiation (*two-way anova, $F_{(3,64)}$ = 196.818, p = .000*) treatments, as shown in Fig. 2. Young's modulus of controlled FHAp/FGel composite was measured at 321.04±78.54 MPa, which was decreased to lowest value of 188.45±80.85 MPa after its matrix was blended with PVA at FGel/PVA percent mass ratio of 7.5/2.5 (w/w). However, it was increased again at PVA content of 5.0 and 7.5 % (w/v) to 454.21±164.47 and 783.75±183.56 MPa, respectively. This condition is related to flexible nature of PVA at low concentration but it is stiffer as concentration is increased [25]. Highest Young's modulus was observed at 2057.42±176.22 MPa for composites treated with combination of highest PVA content and highest irradiation dose showed highest value. Significant interaction (*two-way anova, $F_{(9,64)}$ = 3.388, p = .002*) was occurred between both treatments. Regression analysis revealed that the slope of curves *irradiation dose vs compressive strength* were at 18.283 (R^2=0.99), 31.378 (R^2=0.99), 28.755 (R^2=095), and 28.561 (R^2=0.95) for FGel/PVA percent mass ratio of 10/0, 7.5/2.5, 5.0/5.0, and 2.5/7.5 (w/w), respectively. In similar to regression analysis of compressive strength at yield, this results indicated that crosslinking reaction induced by gamma irradiation was less effective at higher PVA content.

Adequate compressive strength are generally required for bone scaffold to withstand external stress and maintain its integrity. Reference [26] reported that scaffold with minimal compressive strength and 0.05-1 GPa of Young's modulus are required for low load-bearing applications, while more than 50 Mpa strength and more than 1 Gpa of Young's modulus for medium load-bearing application. For High load-bearing application, scaffold with more than 150 Mpa strength and 10-30 Gpa of Young's modulus is prerequisite. Based on its compressive strength, FHAp/FGel based composites in this study was in interval which is suitable for low load-bearing application only.

B. FTIR Spectra

FTIR spectra of FGel and FHAp showed characteristic peaks related to the presence of specific functional group i. e. amide group of polypeptide and phosphate group of calcium phosphate compound, respectively, as shown in Fig. 3.

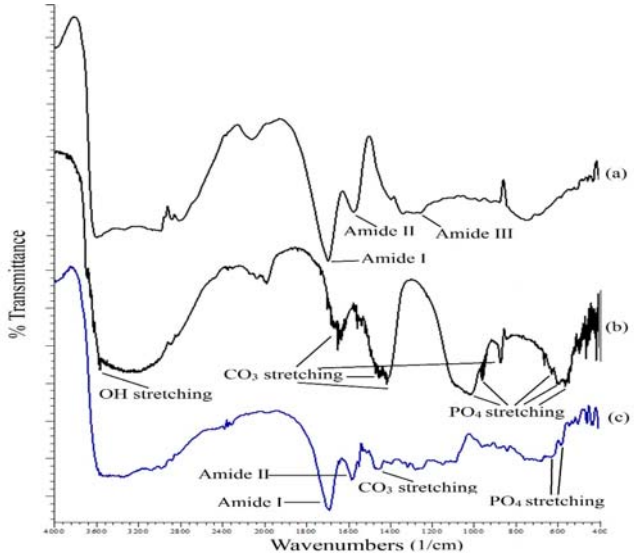

Fig. 3 FTIR spectra of (a) FGel, (b) FHAp, and (c) non-irradiated FHAp/FGel composite

FTIR spectrum of non-irradiated FHAp/FGel composite showed characteristic peaks of both FHAp and FGel. As compared to FGel, amide I peak of composite was blue shifted to 1689 cm^{-1}, while amide II was red shifted to 1577 cm^{-1}. As compared to FHAp, the structural OH stretching peak was disappeared, while PO_4 stretching peaks was red shifted to 631 and 586 cm^{-1}. Shifting pattern of the peaks was in accordance to previous studies which indicates the inorganic-organic (Ca^{2+}-COO^-) bonds between FGel and FHAp [30, 31].

The characteristic IR peaks of PVA were a broaden peak between 3500-3000 cm^{-1} attributed to hydrogen-bonded O-H stretching, and peak at 2946 cm^{-1} attributed to aliphatic C-H stretching, as shown in Fig. 4. Peaks at 1713 and 1576 cm^{-1} were attributed to C=O stretching vibration band from carbonyl ether due to the residual acetate remaining after the preparation of PVA from hydrolysis of polyvinyl acetate. Residual acetate also contributed to peaks at 1143 and 1097 cm^{-1} which attributed to C-O-C stretching and C-O stretching group, respectively [25].

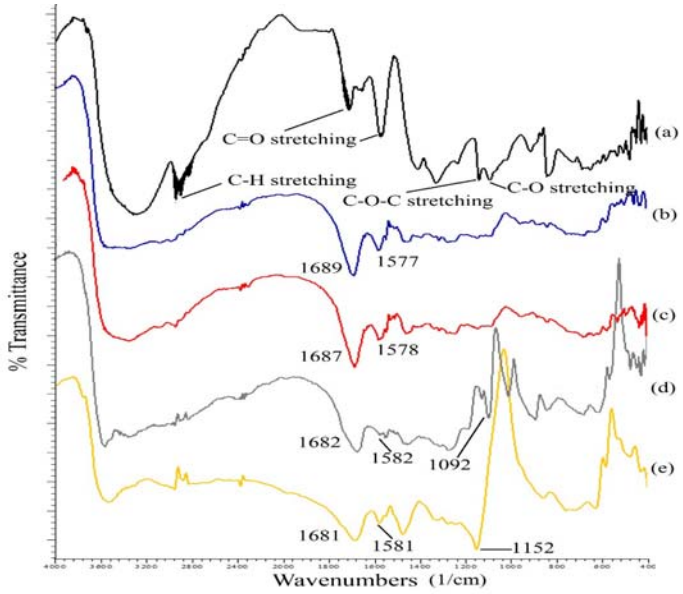

Fig. 4 FTIR spectra of non-irradiated of (a) PVA, and FHAp/FGel-PVA composite at FGel/PVA percent mass ratio of (b) 10/0, (c) 7.5/2.5, (d) 5.0/5.0, and (d) 2.5/7.5 (w/w)

Alteration to FTIR spectra of FHAp/FGel based composite was observed on scaffold with FGel/PVA mass ratio at 5.0/5.0 and 2.5/7.5, respectively. Sharp peak at 1092 cm^{-1} attributed to C-O stretching was observed at FGel/PVA mass ratio of 5.0/5.0, while sharp peak at 1152 cm^{-1} attributed to C-O-C stretching was observed at FGel/PVA mass ratio of 2.5/7.5. As compared to FHAp/FGel composite, wavenumbers of amide I peak and amide II were further blue shifted and red shifted, respectively, which indicates formation of hydrogen bonds between amine (NH) groups in FGel and hydroxyl (OH) group in PVA [30,31].

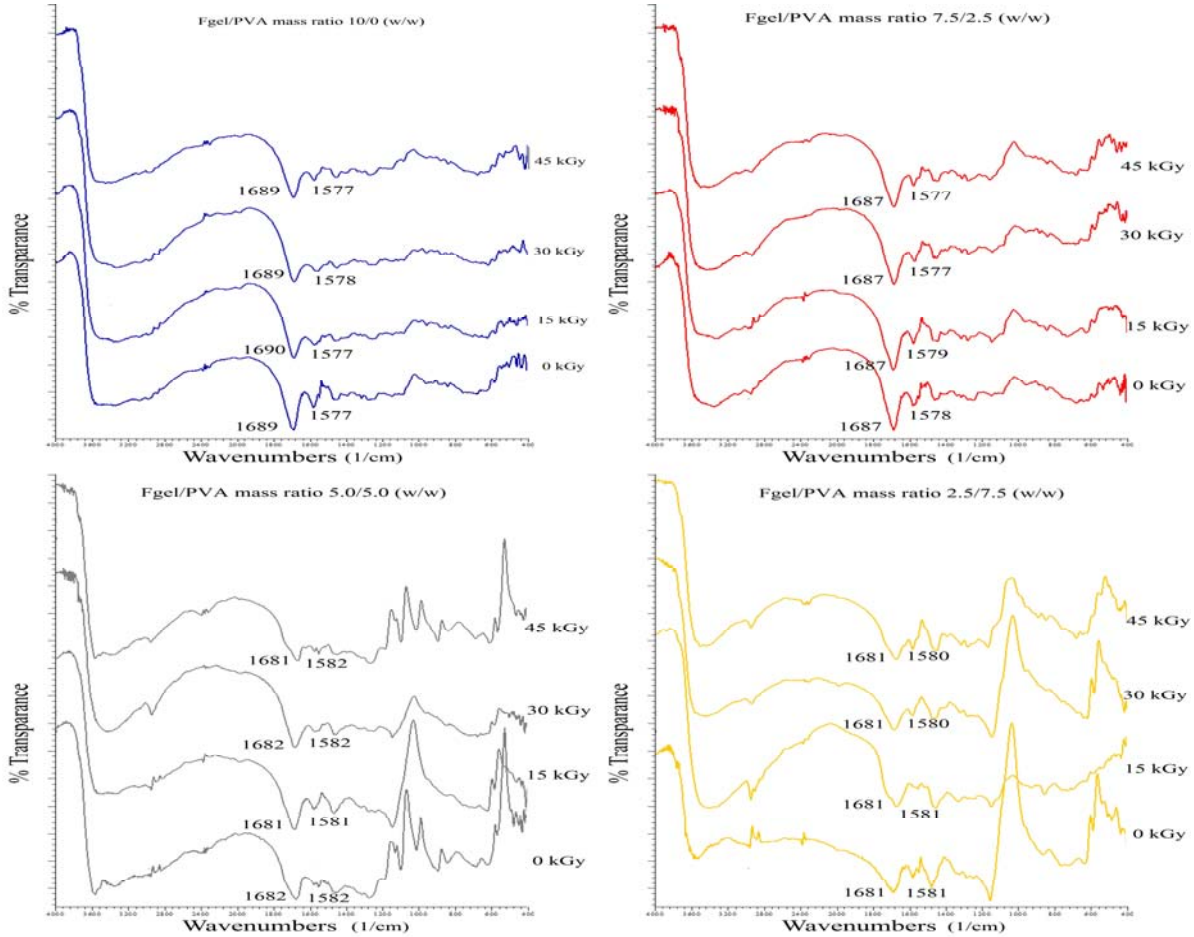

Fig. 5 FTIR spectra of irradiated FHAp/FGel based composite at varied FGel/PVA percent mass ratio

Characteristic peaks of FHAp/FGel based scaffolds were not changed related to gamma irradiation treatment, as shown in Fig. 5. It seems that amide bond in gelatin. Several references [32,33] reported that crosslinking sites in gelatin molecule under ionizing radiation are at amino acid side chain of tyrosine, phenylalanine, histidine, methionine, and cysteine. Gamma irradiation also abstract hydrogen atom in C-alpha of in gelatin main chain [34]. While, radical sites for crosslinking in PVA chains are at C-alpha and C-beta due to H-abstraction reaction. Transformation of PVA radicals to ground state may involving transformation OH groups to be ether groups [35]. However, hydroxyl groups are abundantly occurs in side chain of PVA, therefore characteristic peaks of PVA may not much changed.

C. SEM Analysis

Pores in the scaffold are the void that left after sublimation of water in solvent-rich region during freeze-drying [36]. Macropores (pore with size 50 – 200 μm) and/or capillaries (pore with size 50 – 200 μm) are key factor for cell attachment and proliferation within scaffold matrix [37]. The SEM micrographs of irradiated FHAp/FGel based composites were shown in Fig. 6 and Fig. 7, respectively.

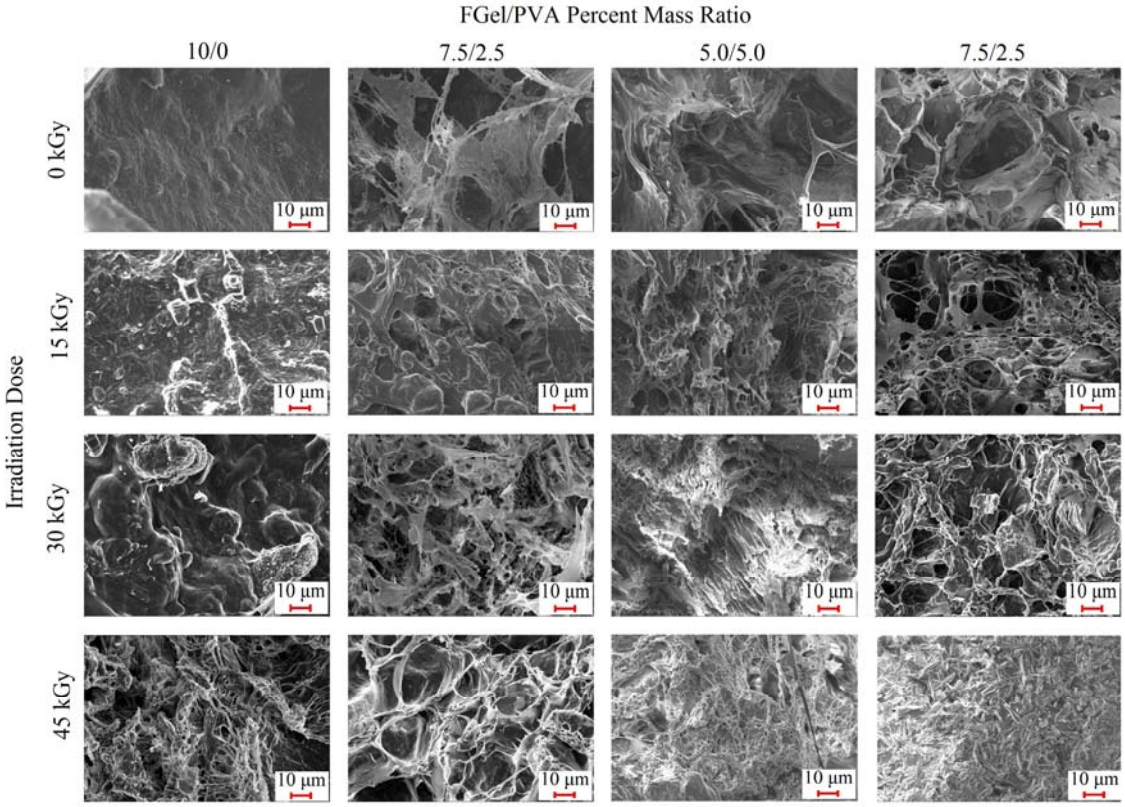

Fig. 6 SEM micrograph of surface roughness of irradiated FHAp/FGel based composite at varied FGel/PVA percent mass ratio and irradiation doses

As shown in Fig. 6, inclusion was not observed in the matrices of composites. It seems that FGel-PVA blend and FHAp filler were homogenously dispersed in scaffold. Smooth film surface was observed for controlled FHAp/FGel scaffold, while fibrous and ribbon structure over film surface were observed in composite whose matrices blended with PVA. The fibrous and ribbon structure were thick at higher PVA content. The surface roughness was increase at higher irradiation dose due to formation of pore at size up to 20 μm (mesopores). Reference reported that a rough surface may imprison the fibrin matrix better than a smooth surface, and hence facilitate the migration of osteogenic cells to the materials surface [38]. Further, the fracture on the surface of scaffold with FGel/PVA percent mass ratio of 2.5/7.5 (w/w) irradiated at 45 kGy was observed to forming a non-oriented needles-shaped structure.

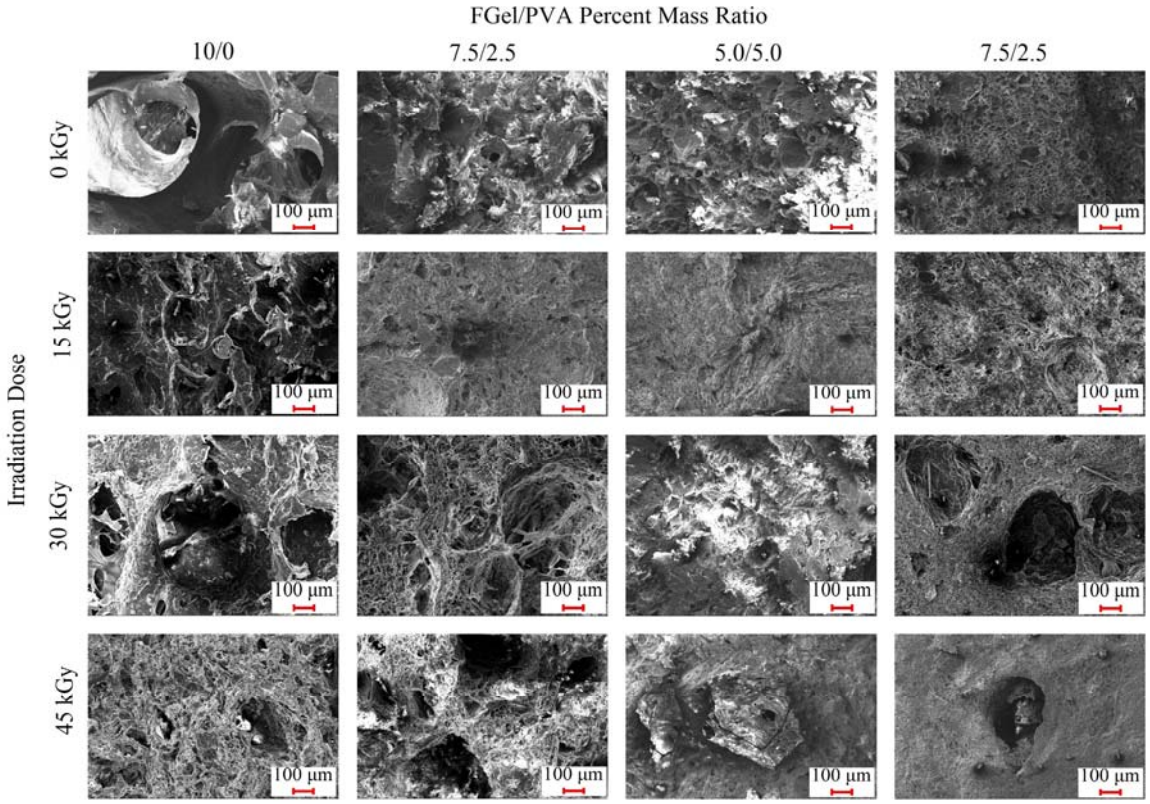

Fig. 7 SEM micrograph of pores of irradiated FHAp/FGel based composite at varied FGel/PVA percent mass ratio and irradiation doses

A must have property for scaffold is interconnected porosity where pore size should be at least 100 µm in diameter for successful diffusion of essential nutrients and oxygen for cell survivability [39]. However, pore size in the range of 200 to 350 µm are found to be optimum for bone tissue in growth [40]. As shown in Fig. 7, composites with pore size of > 50 µm (macropores and/or capillaries) was only observed for FGel/PVA percent mass ratio of 10/0 and 7.5/2.5 (w/w). Lack of macropores at composite with high PVA content was related to excellent film forming capacity of PVA which prevent the phase separation during freeze-drying. However, macropores may be obtained if additional treatment such as freeze thawing cycles is subjected to PVA-based hydrogels [41]. Furthermore, the increase of irradiation dose lead to formation of micro- and mesopores in the wall surface of composite. References reported [42] that multi-scale porous scaffold involving both micro and macro porosities can perform better than only macroporous scaffold.

IV. CONCLUSIONS

Based on this study, FHAp and FGel from scale of *Lates calcarifer* Bloch can be used to synthesis composite as candidate of bone scaffold. PVA blending and gamma irradiation were significantly improved compressive strength of FHAp/FGel composites. Both treatments may increase surface roughness of FHAp/FGel based composite by formation fibrous and ribbon structure. The FHAp/FGel based composites in this study was suitable for low load-bearing application only. Multi-scale pore size was observed in irradiated FHAp/FGel based composite with FGel/PVA percent mass ratio of 10/0 and 7.5/2.5 (w/v).

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