POLYMERIC BIOMATERIALS FILM BASED ON POLY(VINYL ALCOHOL) AND FISH SCALE COLLAGEN BY REPETITIVE FREEZE-THAW CYCLES FOLLOWED BY GAMMA IRRADIATION

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ABSTRACT

The polymeric biomaterial film based on poly(vinyl alcohol) (PVA) and fish collagen of Lates calcarifer scale were synthesized by three times freeze-thaw cycles followed by gamma irradiation at varied doses of 0, 10, 20, and 30 kGy respectively. Characteristic of PVA/collagen film as effect of constituting polymers and cross linking methods were investigated using Fourier Transform Infrared (FTIR) spectrophotometer, Differential Scanning Calorimetry (DSC), Universal Testing Machine (UTM), and Chroma Mater. Its gel fraction and swelling kinetic were determined by gravimetry. The result showed that IR spectra of controlled and irradiated film demonstrated characteristic vibration bands of both constituting materials. The DSC analysis revealed that gamma irradiation induced interaction between PVA and collagen at molecular level. Improvement of tensile properties by gamma irradiation was observed on tensile strength at 30 kGy with $p < 0.05$. Gamma irradiation also significantly ($p < 0.05$) increased yellowness of PVA/collagen film, reduced swelling kinetic, and increased gel fraction of films.

Keywords: *polymeric biomaterial film; poly(vinyl alcohol); fish collagen; gamma irradiation*

ABSTRAK

Film biomaterial polimer berbasis poli(vinil alkohol) (PVA) dan kolagen ikan dari sisik Lates calcarifer disintesis melalui tiga siklus beku-leleh dilanjutkan iradiasi gamma pada variasi dosis 0, 10, 20, dan 30 kGy. Karakteristik film PVA/kolagen sebagai efek materi penyusun dan metode sintesis diinvestigasi menggunakan spektrofotometer Fourier transfrom inframerah (FTIR), kalorimeter pemindaian diferensial (DSC), mesin uji universal (UTM), dan Chroma meter. Kinetika swelling dan fraksi gel ditentukan secara gravimetri. Hasil menunjukkan bahwa spektrum IR film kontrol dan iradiasi menunjukkan karakteristik band vibrasi kedua bahan penyusun. Analisis DSC menunjukkan bahwa iradiasi gamma menginduksi interaksi PVA dan kolagen pada tingkat molekul. Iradiasi gamma pada dosis 30 kGy secara signifikan ($p < 0,05$) menyebabkan peningkatan kekutan tarik film. Iradiasi gamma juga secara signifikan ($p < 0,05$) menyebabkan peningkatan intensitas warna kekuningan, penurunan kinetika pengembangan, dan peningkatan fraksi gel film PVA/kolagen.

Kata Kunci: *film biomaterial polimer; poli(vinil alkohol); kolagen ikan; iradiasi gamma*

INTRODUCTION

Polymeric biomaterial is a term to designate a new class of materials based on blending synthetic and natural polymers for biomedical applications. Originally, these new materials were conceived to overcome the poor biological performance of synthetic polymers and also to enhance the mechanical characteristics of biopolymers, in order to be employed as biomaterials or as low-environmental impact materials [1-3]. Collagen and poly(vinyl alcohol) (PVA) are ones of among combination that been used as polymeric biomaterials in a wide range of applications in forms of hydrogels, films, and sponges [4-5].

The concept in PVA/collagen blend is to incorporate a slowly degradable, bio-inert PVA of high glass transition temperature and biodegradable bioactive natural macromolecule collagen in a system [6]. PVA constitute a harsh environment which are incompatible with cells and tissue. Improvement in the characteristic of PVA can be achieved by blending with collagen, the major component in natural extra cellular matrix [7]. Collagen substrate can modify the morphology, migration and differentiation of cells [8]. These properties of collagen emphasize its significance in tissue regeneration and its value as a scaffold material. By blending with PVA, weak properties of collagen such as fast biodegradation rate and poor mechanical strength can be overcome [9].

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Previous study of collagen and PVA blend [10-12] showed that the collagen/PVA blends also display good film-forming properties which are a favorable characteristic for application in biomedical field. The drawbacks of the blend are the immiscibility of PVA and collagen solutions except for a very low concentration of PVA in which they interact by hydrogen bonds, and their poor mechanical properties [11]. A further improvement in the characteristics of such materials could reasonably be expected if interaction that stronger than hydrogen bonds occurred between collagen and PVA [6].

To support this purpose, films have been prepared from PVA and cross-linked by a two-stage process of repetitive freeze-thaw cycle followed by gamma irradiation. Collagen was collected by acid extraction at low temperature from Baramundi fish (*Lates calcarifer*) scales. Fish collagen was used due to *halal* issue in Islam religion and prevention of potential transfer of virus related infectious disease as in mammalian sourced collagen [13]. These two steps cross linking approach were applied because this approach does not introduce potentially cytotoxic chemical residues as in the case with chemical approach [14-15]. Moreover, radiation treatment is effective in sterilizing biomaterials [16]. As all properties of the polymeric materials are dependent on the constituting materials and cross linking method adopted, it is important to determine the alteration of properties of polymeric biomaterials film based on PVA and fish scale collagen of *Lates calcarifer* scales that resulted by freeze-thaw processing followed by gamma irradiation at dosis 0, 10, 20, and 30 kGy.

EXPERIMENTAL SECTION

Materials

Collagen was extracted from scales of sea fish *Lates calcarifer* by dissolution in 0.5 M acetic acid at biomaterial lab in Center of the Application of Isotopes and Radiation Technology (CAIRT), National Nuclear Energy Agency, Republic of Indonesia. Dry collagen is in white powder form, insoluble in water and soluble in acid. The PVA (Merck, MW = 7200) and citric acid monohydrate (Merck, MW = 210.14) were of analytical grade and used without further purification. Water used in whole experiment was aqua bidest.

Instrumentation

The chemical changes of PVA/collagen film were analyzed using Fourier Transform Infrared (FTIR) IR-Prestige-21 spectrometer model 800 series, Shimadzu. Thermal properties of films were determined using DSC-60, Shimadzu. Tensile strength, yield strength and elongation at break of films were measured using Universal Testing Machine Strograph-R1, Shimadzu. Color differences of irradiated films were measured using Chroma Meter CR-200b, Minolta. Sample was irradiated in Center for the Application of Isotopes and Radiation Technology using Gamma Irradiator "*Karet alam*" from cobalt-60 source with an activity of 118.6 kCi in September 2008.

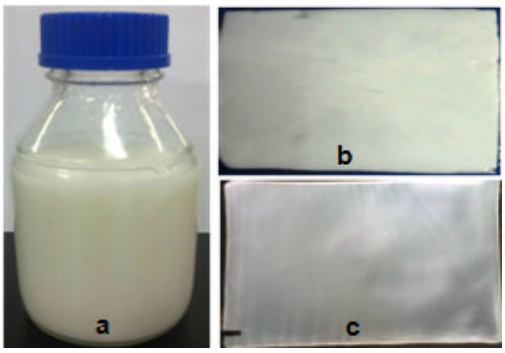

Fig 1. PVA/collagen blend sample in the forms of (a) solution, (b) hydrogels, and (c) film

Procedure

Preparation of irradiated PVA-collagen films

PVA/collagen blend were prepared by mixing 10% PVA solution in water and 10% (w/v) collagen solution in 0.5 M citric acid at ratio 1:1. Solution was casted onto polypropylene plate and subjected to 3 times freeze-thaw cycles to form hydrogels. One freeze-thaw cycle was defined as 16 h freezed condition at -20 °C followed by 8 h thawed condition at 25 °C. Then, hydrogels were rinsed with water to remove acidic solvent and irradiated by gamma rays of cobalt-60 at varied doses of 0, 10, 20, and 30 kGy respectively. PVA/collagen films were obtained by drying control and irradiated hydrogels in air at room temperature. The films were easy to handle and its thickness was 0.1714 ± 0.0459 mm. Samples of PVA/collagen blend solution, hydrogel, and film were shown in Fig. 1.

FTIR characterization

FTIR was used to characterize the presence of specific chemical groups in films. FT-IR spectra were obtained over the range of 4000-400 cm^{-1}. Samples were milled and mixed with dried KBr powder placed in a sampling cup, 20 scans were required at 2 cm^{-1} resolution with subtraction of the KBr background. FT-IR spectra were also obtained for pure PVA and dry collagen for comparison with samples.

DSC characterization

A thermal analysis instrument (DSC-60, Shimadzu) was used to determine the thermal

properties of films. Samples were conditioned at 20% relative humidity for 24 h prior to DSC characterization. The amount of sample to measure DSC spectra was 5.0 ± 0.1 mg. Each sample was heated on non-sealed platinum pan from 23 °C to 300 °C in air at a rate 10 °C/min. The use of non-sealed sample pan in DSC measurement was due to allow water evaporation so the plasticizer effect of water vapor expected to be minimized.

Tensile test

Tensile properties of films such as tensile strength, yield strength and elongation at break were measured using Universal Testing Machine (Strograph-R1, Shimadzu). Samples were moulded using dumbbell ASTM-0-1822-L for the preparation of the standard size measurement. The crosshead speed was set at 25 mm/min. The resultant data was showed at the recorder. The elongation at break was defined by Eq. 1:

$$Elongation\,at\,break = \frac{L_i - L_0}{L_0} \times 100 \quad (1)$$

in which L_0 is the original length and L_i is the final length. The tensile strength was defined by Eq. 2:

$$Tensile\,strength = \frac{F_{max}}{A} \quad (2)$$

in which F_{max} is the amount force of exerted while stretching a sample until the samples fails (kg), and A is the area of the samples (cm^{-2}). And the yield strength was determined by 0.2% off-set method and defined by Eq. 3:

$$Yield\,strength = \frac{F_{\sigma y(0.2\%)}}{A} \quad (3)$$

in which $F_{\sigma y(0.2\%)}$ is the amount force of exerted at the point where a parallel line to the elastic region intersects with the stress-strain curve (kg), and A is the area of the samples (cm^{-2}).

Color test

Colors changes of PVA/collagen films were measured using a colorimeter (Chroma Meter CR-200b, Minolta). The sample was placed on a white plate and the CIE 1976 color scale was used to measure relative color change: L^* = 0 (black) to L^* = 100 (white); a^* = -80 (greenness) to a^* = 100 (redness); and b^* = -80 (blueness) to b^* = 70 (yellowness).

Gel fraction and swelling test

Gel fraction of film was measured by immersing the film in a water bath for 48 h at 37˚C. The swollen film was then placed in a 60˚C oven for 2 days to completely dry the samples. The gel fraction was defined by Eq. 4:

$$Gel\,fraction(\%) = \frac{m_d}{m_0} \times 100 \quad (4)$$

in which m_d is the weight of oven-dried temperature of swollen film, and m_0 is the initial weight of film.

Isothermal degree of swelling (*SD*) of films was determined as a function of times. Film was swollen in water at 37 °C to achieve the equilibrium swelling degree. The degree of swelling of films was measured after 5, 10, 15, 20, 30, 40, 60, 90, 120, 150, 180, and 1080 min respectively. The degree of swelling was defined by Eq. 5:

$$SD(\%) = \frac{m_t - m_0}{m_0} \times 100 \quad (5)$$

in which m_t is the weight of swollen film at time t, and m_0 is the initial weight of film.

The equilibrium swelling degree (SD_{eq}) is the degree of the swollen film at equilibrium in which the swollen film had reached constant mass (m_{eq}).

Statistical analysis

All the statistical analysis was performed by One-Way ANOVA with significance level defined at $p < 0.05$ followed by Tukey's Honestly Significant Difference (HSD) test if ANOVA test indicates differences among groups in sample.

RESULT AND DISCUSSION

FTIR Characterization

The FTIR transmittance spectra of fish collagen, PVA, and PVA/collagen at varied irradiation doses were shown in Fig. 2. The blend polymers have most of PVA characteristic IR absorption bands. The characteristic IR peaks of film contributed by PVA were a peak centered at 3543 cm^{-1} attributed to O-H stretching, and also two peaks at 2955 cm^{-1} and 2861 cm^{-1} attributed to characteristic bands of asymmetric and symmetric C-H stretching. A peak at 1726 cm^{-1} was attributed to C=O stretching vibration band from carbonyl functional groups due to the residual acetate groups remaining after the preparation of PVA from hydrolysis of polyvinyl acetate or oxidation during preparation and processing [17-18].

In addition to PVA peaks, three new absorption peaks appeared at 3615 cm^{-1}, 1650 cm^{-1} and 1580 cm^{-1}, which are typical to infrared features of collagen namely amide A, amide I and amide II bands [19]. Generally, amide A originates from N-H stretching vibration, the amide I band originates from C=O stretching vibration coupled to N-H bending vibration and amide II band arises from N-H bending vibration coupled to C-N stretching vibration of the peptide linkage [19-20].

Compared to pure polymers, the C-H stretching peaks was stronger related to inference of C-H content

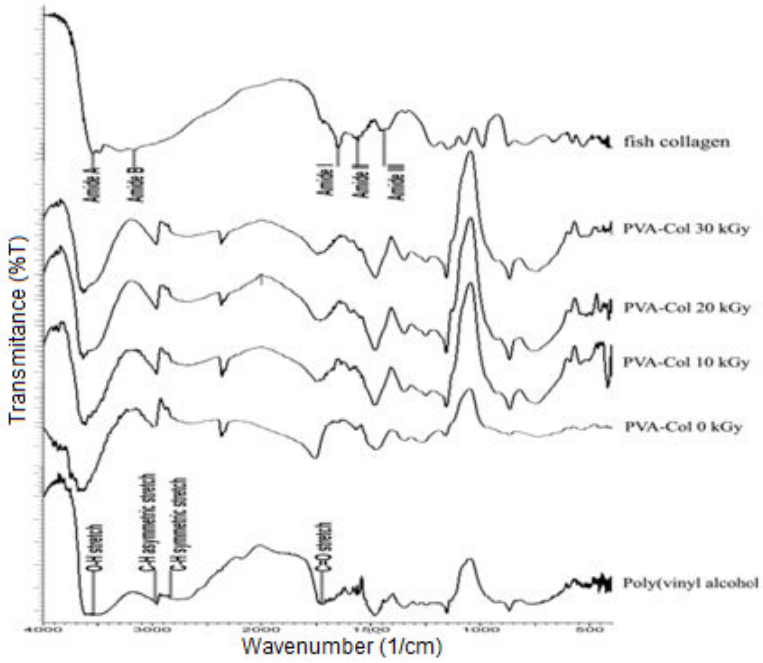

Fig 2. FTIR spectra of fish collagen, PVA, and PVA-collagen at varied irradiation doses

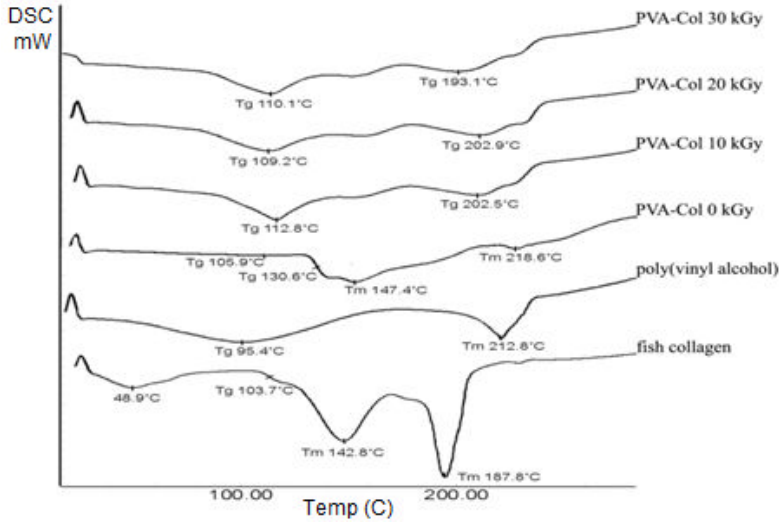

Fig 3. DSC curves of fish collagen, PVA, and PVA-collagen at varied irradiation doses

of PVA and collagen. The PVA/collagen blend gave strong O-H stretching peak, and very weak amide I and II peak indicating the occurrence of physical cross linking between hydroxyl groups of PVA with amide group of collagen through hydrogen bond [21]. This interaction was red-shifted amide I band from a 3553 cm^{-1} in pure collagen to a 3615 cm^{-1} in PVA/collagen film due to closer hydrogen bond length.

Gamma irradiation was observed to influencing FTIR spectra of PVA/collagen film specifically on amide I and II band. Both amide I and amide II peaks of irradiated film were gradually disappeared related to higher radiation dose indicating the loss of secondary structure of collagen [19]. Secondary structure of α-helix is known greatly reduced under irradiation while less damage occurred on the β-sheet structure [22-24].

DSC Characterization

DSC characterization had been carried out to get some information about transition temperatures of the prepared samples and it is one of the most convenient methods to determine miscibility and thermal properties of polymer blend [28-29]. The DSC curve (first scan) of collagen, PVA, and PVA/collagen at varied doses was shown in Fig. 3.

Table 1. Tensile properties of PVA/collagen films irradiated at different irradiation doses

Irradiation dose (kGy)	Elongation at break (%)	Tensile strength (kg/cm^2)	Yield strength (kg/cm^2)
0	202.22 ± 35.28^a	1500.91 ± 150.59^a	1480.99 ± 214.10^a
10	205.56 ± 13.33^a	1514.89 ± 220.80^a	1430.31 ± 140.30^a
20	204.44 ± 19.44^a	1558,90 ± 234.99^a	1391.64 ± 215,69^a
30	203.33 ± 40.93^a	1939.04 ± 262.62^b	1558.62 ± 187.54^a

In each column, the differences between means followed by a same superscript letter were not significant (Tukey's test, p = 0.05, n = 9)

It can be observed that DSC spectra of PVA, collagen and PVA/collagen film showed characteristic features of semicrystalline materials in which the baseline shift related to glass transition (*Tg*) is prior to endothermal peak of melting point (*Tm*). The *Tg* is the melting of disordered/amorphous region, and the *Tm* is the melting of crystalline region [30-31]. DSC curve of pure PVA showed two endothermal peaks that signed as *Tg* and *Tm*.

The DSC curve of dry collagen showed an endothermal peak at 48.98 °C related to the release of free water from the matrix, a baseline shift of the glass transition (*Tg*) of a disordered fraction originated by partial denaturation of collagen, and two endothermal peaks (*Tm*) of melting/denaturation of collagen. The first *Tm* that follows the transition is attributed to a thermal history effect associated with room temperature ageing and the second *Tm* was related to irreversible denaturation of collagen structure, which also referred as denaturation point (*Td*) [32-33]. Upon denaturation, collagen fibrils undergo several conformational changes caused by the breaking of different cross-links present at the intermolecular level and release of the H-bonded water used that stabilize the triple helix structure of collagen molecule. The end result of the thermal denaturation of collagen is random fragmentation of the collagen fibril and molecule [34-35].

The calorimetric results on physically cross linked PVA/collagen blends have shown that these two polymers were thermodynamically immiscible. These results are in agreement with previous studies [2,6,11,21]. The thermal transition of blend was those typical of the pure polymers with two *Tg* at 105.9 and 130.5 °C. It is concluded that the binary blends examined are heterogeneous systems, composed of pure polymer phases. However, homogeneity of the blend suggested continuity of the hard phase throughout the sample so the blend can be classified as mechanically compatible blends [2,6].

In consistent with FTIR spectra, DSC curve of irradiated films evidenced the improvement on blend miscibility caused by chemical cross linking reaction among constituting polymers. As shown in Fig. 3, the DSC curve of irradiated PVA/collagen film showed a single *Tg* or melting peak indicating the component polymers would interact with each other at molecular level [6].

Chemical cross linking within PVA/collagen blend by gamma radiation is proposed through a complex mechanism. The major mechanism is involving the immediate effect of irradiation i.e. radiolysis of water and formation of polymer free radicals [25-27]. These macroradicals mainly produce by indirect effect of radiation while OH radicals attack the polymers to produce the alpha-carbon radical peptide in case of collagen. For PVA, the OH radicals can either abstract an H atom in α-position to the OH group (70%) or at the neighboring methylene group (β-position, 30%). The main reaction that involved OH groups may be the underlying reason of strong O-H stretching band in IR spectra of irradiated film blend. Further, mutual recombination of these macroradicals induces formation of intra- and intermolecular cross linking and semi- and interpenetrating network polymers [26-27].

There also can be noticed for the values of *Tg* and *Tm* of irradiated PVA/collagen blend were in between of pure polymers. The *Tg* was slightly increased at around 110 °C and the *Tm* of irradiated film blend was decreases at around 200 °C. Both changes was caused by the increased of HC content contributed by collagen. The *Tm* of irradiated film was observed become lower at higher irradiation dose. This result suggested as the result of the formation of some imperfections on crystalline region by gamma irradiation [36].

Effect of Irradiation on Tensile and Color Properties

Polymeric biomaterials films may be subjected to various kinds of stress during routine application. The study of the mechanical properties is of primary importance for determining the performance of the materials [37]. Tensile properties of PVA/collagen film related to cross linking method was shown in Table 1.

Based on Table 1, the tensile properties value of films indicates its ability to withstand with stress and strain. The tensile properties of films exhibit deformation of plastic polymer. Under tensile stress, films undergone elastic deformation followed by plastic deformation before fracture. Elastic deformation was observed as yield strength in which stress proportional to strain in small strain. This stage deformation is reversible. Plastic deformation was observed as elongation and tensile strength at high strain in which

Table 2. The dose effect of gamma irradiation on color change of PVA/collagen films

Irradiation dose (kGy)	Hunter color values		
	*L**	*a**	*b**
0	84.0 ± 0.2[a]	20.6 ± 0.1[a]	- 0.2 ± 0.3[a]
10	84.1 ± 0.4[a]	20.6 ± 0.2[ab]	0.7 ± 0.2[b]
20	84.0 ± 0.3[a]	20.4 ± 0.1[bc]	1.2 ± 0.6[bc]
30	83.9 ± 0.2[a]	20.3 ± 0.1[c]	1.8 ± 0.7[c]

In each column, the differences between means followed by a same superscript letter were not significant (Tukey's test, p = 0.05, n = 12)

Table 3. Gel fraction and swelling kinetic parameter of irradiated PVA/collagen film

Radiation dose (kGy)	Swelling kinetic parameter					Gel Fraction (%)
	t_{in} (min)	v_{in} (min^{-1})	t_{eq} (min)	v_{eq} (% min^{-1})	SD_{eq} (%)	
0	20	14.06[a]	150	2.24[a]	335.2204[a]	45.49 ± 2.16[a]
10	20	13.97[a]	180	1.77[b]	317.8762[b]	51.60 ± 3.08[b]
20	20	12.89[ab]	180	1.71[b]	308.1198[b]	71.94 ± 6.70[c]
30	20	12.22[b]	180	1.53[c]	275.4032[c]	79.64 ± 2.69[d]

In each column, the differences between means followed by a same superscript letter were not significant (Tukey's test, p = 0.05, n = 10)

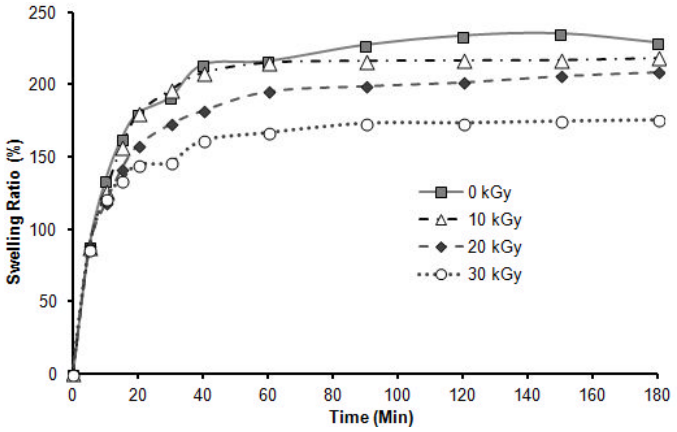

Fig 4. The isothermal swelling of irradiated PVA/collagen film in function of time

stress increases very slowly with increased strain in small strain followed by fracture. This stage deformation is irreversible [38-40].

Gamma irradiation was observed not significantly affect the values of elongation at break and yield strength of films. Significance difference only occurred on tensile strength when film was irradiated at 30 kGy ($p < 0.05$). The increase on tensile strength was assumed as the effect of covalent bond formation within network structure in irradiated film [6,36,41].

The color properties of films were affected by irradiation doses as shown in Table 2. The Hunter color *a**-value was increased and *b**-values was decreased related to higher irradiation doses up to 30 kGy. The *L**-value, related to transparency of film, was not significantly change. This result indicates that when the irradiation is applied for a better mechanical property, the transparency and yellowness of the films should be considered.

Swelling Kinetic and Gel Fraction

The isotherm swelling curves of films were showed similar shape both in controlled and irradiated film, as shown in Fig. 4. Several parameters such the initial swelling time (t_{in}), the initial swelling degree (SD_{in}), the initial swelling rate (v_{in}), the equilibrium swelling time (t_{eq}), the saturation swelling rate (v_{eq}), and equilibrium swelling degree (SD_{eq}) were determined based on Fig. 4 to observe the effect of irradiation dose on swelling kinetic of films. The v_{in} is the rate of swelling during initial linear region of swelling as determined by Eq. 6 [42].

$$v_{in} = \frac{SD_{in}}{t_{in}} \quad (6)$$

in which the t_{in} is defined as the time interval within which the degree of swelling increase linearly with swelling time from, and the SD_{in} is the degree of swelling at the end of this linear increase. The v_{eq} is the rate of swelling when equilibrium swelling degree is attained at the first time as determined by Eq. 7 [42].

$$v_{eq} = \frac{SD_{eq}}{t_{eq}} \quad (7)$$

in which the t_{eq} is the time when the equilibrium degree is first attained, and the SD_{eq} is the saturation swelling rate. The parameter was shown in Table 3.

In consistent with previous data, swelling kinetic parameters also indicated the increase of cross linking density at higher radiation dose within PVA/collagen film. It is observed that the initial swelling rate, swelling rate at equilibrium, and the equilibrium degree of swelling decreased related to higher irradiation dose. As cross linking density increase, sites to form hydrogen bonds with water are less available which reduce the water content [43]. It also decreased the

pore size of the network which decreased the swelling ability due to the slower relaxation time of the polymeric chains [44-45]. The increase on cross linking density was evidenced by gel fraction determination of irradiated film that was significantly higher at higher radiation dose ($p < 0.05$). Regardless the cross linking density, no difference was observed in initial swelling time. It means that the free molecules and free water can always move within the structure through pores and be released out of the swelled films [46].

CONCLUSION

Polymeric biomaterial film based on poly(vinyl alcohol) and fish collagen has been synthesized by repetitive freeze-thaw cycles followed by gamma irradiation. This two-step cross linking technique provides advantages for application in biomedical field. Repetitive freeze-thaw cycle caused physical cross linking to forming a hydrogels so that convenient to handle and easily shaped into desirable forms. Gamma irradiation modified interaction among constituting polymer that furthermore modified its physicochemical properties of blend through formation covalent bond. This method is preferable for application in biomedical field because it provides convenient preparation, does not introduce potentially cytotoxic chemical residues, and sterilized the material.

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