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Original Article

Poly(lactic acid)/chitosan foams prepared by conventional method: Mechanical properties, cytotoxicity and *in-vitro* degradation

Pasuta Sungsee and Varaporn Tanrattanakul*

Department of Materials Science and Technology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand 90110

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Abstract

The present study evaluated the feasibility of using a conventionally prepared poly(lactic acid) (PLA) foam. A PLA foam was successfully prepared by compression molding. Azodicarbonamide was used as a chemical blowing agent. Chitosan was added as a bioactive material and poly(ethylene glycol) (PEG) was added as a plasticizer for PLA. Chitosan promoted the plasticization effect of PEG. The combination of chitosan and PEG contributed the lowest T_g and T_{cc} of PLA. The plasticized PLA showed greater impact strength than PLA. All foams showed closed-cell morphologies with relatively large pores. Chitosan and PEG reduced pore size and the reduction in pore size was greater when both additives were combined. Cytotoxicity was determined from MG-63 cell proliferation on samples, and all foams showed non-cytotoxicity. The addition of chitosan and/or PEG decreased weight loss of PLA in *in-vitro* degradation tests. This work was preliminary work and more studies need to be done.

Keywords: poly(lactic acid), chitosan, foam, tissue engineering scaffold, cytotoxicity

1. Introduction

Tissue engineering scaffolds are three-dimensional, artificial, porous biomaterials used in tissue culturing and are designed to induce cell adhesion, cell differentiation, cell proliferation and tissue formation both *in-vitro* and *in-vivo* (Chen-Turng *et al*., 2015). Tissue engineering scaffolds have been prepared from poly(lactic acid) (PLA) by several techniques. These techniques included 3D printing (Serra, Planell, & Navarro, 2013), solid freeform fabrication (Tanodekaew, Channasanon, Kaewkong, & Uppanan, 2013), solvent casting with particle leaching (Huang, Zhu, Zhao, & Wan, 2014), thermally induced phase separation (La Carrubba, Carfì Pavia, Brucato, & Piccarolo, 2008), batch foaming with supercritical CO_2 (Chen-Wen *et al*., 2014) and a combination of batch foaming with supercritical CO_2 and particle leaching (Chen-Turng *et al*., 2015). Supercritical CO_2 has been used as a physical blowing agent for PLA foam prepared by extrusion (Pilla, Kim, Auer, Gong, & Park, 2009) or injection molding (Zafar-Ghosh *et al*., 2016). All these techniques require expensive specialized equipment.

PLA foam can be produced by a foaming process that uses a chemical blowing agent in the melt state. An interesting chemical blowing agent is azodicarbonamide (AZDC). It is cheap and foam can be produced using a very simple compression molding technique with inexpensive equipment. Using thermal compression followed by foam expansion without pressure in an electric oven, Zimmermann, Brambilla, Brandalise, and Zattera (2013) prepared PLA foam using AZDC with zinc oxide (ZnO). Luo-Jian *et al.* (2013) successfully prepared PLA foam by a chemical foaming in compression molding process, also using AZDC and ZnO. PLA foam has been produced by extrusion using a commercial blowing agent, BIH40 (Matuana, Faruk, & Diaz, 2009), citric acid and baking soda (Kmetty, Litauszki, & Rèti, 2018). However, to the best of our knowledge, a PLA foam prepared using typical compression molding and AZDC has not been used in a tissue engineering scaffold application.

PLA has several advantages but one of its disadvantages is brittleness; therefore, plasticizer is usually

*Corresponding author
Email address: varaporn.t@psu.ac.th

added to PLA. Poly(ethylene glycol) (PEG) is one of the common plasticizers for PLA. Tissue engineering scaffold material has included chitosan for its biodegradability, biocompatibility, non-toxicity and antimicrobial activity (Bonilla, Fortunati, Vargas, Chiralt, & Kenny, 2013; Cai, Li, Weihs, & Wang, 2017; Rodríguez-Vázquez, Vega-Ruiz, Ramos-Zúñiga, Saldaña-Koppel, & Quiñones-Olvera, 2015).

The present study evaluates the preparation of PLA foam by a chemical melting process that occurs in the compression mold using AZDC as a blowing agent. Chitosan and PEG were used as a bioactive material and plasticizer, respectively. To assess the viability of the obtained PLA foam for scaffold application, we determined foam characteristics, mechanical properties, *in-vitro* degradation and cytotoxicity.

2. Materials and Methods

2.1 Materials

Poly(lactic acid) (PLA 4042D) was produced by NatureWorks LLC (Blair, NE, USA), and it was purchased from PTT Polymer Marketing Co., Ltd., Thailand. Chitosan (94.69% degree of deacetylation) from Thai food and Chemical Co., Ltd. was extracted from shrimp shells and had a particle size of ~100 mesh (~149 μm). Poly(ethylene glycol) (PEG) with an average number molecular weight (M_n) of 6000 g/mol was supplied by Sigma-Aldrich Co., Ltd. Azodicarbonamide (AZDC), a blowing agent, was purchased from Greatchem and Supply Pty., Ltd. Zinc Oxide (ZnO), used as an accelerator of AZDC, was purchased from Kit Phaibun Chemistry Ltd., Part.

2.2 Preparation of PLA/chitosan foams

PLA pellets and chitosan powders were dried in an oven for 12 h before use: PLA at 105 °C and chitosan at 50 °C. PLA compounds were prepared in 2 steps. In the first step, PLA, PEG and chitosan were mixed in a twin screw extruder)Prism®TSE16TC(at a screw speed of 80 rpm. Screw temperatures were 140, 165, and 165 °C in the feed, middle and die zones, respectively. The compounded pellets (the mixture of PLA, PEG and chitosan) were obtained. In the second step, those compounded pellets were mixed again in the extruder with AZDC (1.94 wt%) and ZnO (0.06 wt%). Since it was necessary to prevent foam formation in the extruder, the screw temperatures in the feed, middle and die zones were lowered to 130, 150 and 150 °C, respectively, and the screw speed was increased to 150 rpm. In this condition, an unfoamed extrudate could be obtained. A foam sheet was produced by compression molding for 10 min in a closed mold)130 x 130 x 2 mm^3(at a temperature of 145 °C under a pressure of 150 kg/cm^2. The compositions of the prepared PLA compounds are listed in Table 1. The nomenclature of the PLA compounds was based on concentrations of chitosan and PEG.

2.3 Foam characterization

The glass transition temperature (T_g), cold crystallization temperature (T_{cc}), melting temperature (T_m) and degree of crystallization of all foam samples were determined by DSC analyzer (NETZSCH® DSC200F3) from the first heating scan. A heating scan was performed from 20°C to 200°C at the rate of 10°C/min. The degree of crystallization was determined from the first heating scan thermogram, similarly to work of Zimmermann *et al*. (2013), Litauszki and Kmetty (2018) and Sun *et al*. (2018), in terms of X_{c1} and X_{c2} from equations)1(and)2(, respectively, in which ΔH_{PLA} is the heat of fusion of 100% crystalline PLA (93 J/g) (Jaratrotkamjorn, Khaokong, & Tanrattanakul, 2012), ΔH_m is the melting enthalpy, and ΔH_{cc} is the cold crystallization enthalpy.

Table 1. Blend composition of PLA compounds.

Sample code	Composition (wt%)		
	PLA	PEG	Chitosan
PLA	100.0	-	-
1C0P	99.0	-	1.0
3C0P	97.0	-	3.0
0C5P	95.0	5.0	0.0
1C5P	94.1	4.9	1.0
3C5P	92.2	4.8	3.0

$$X_{c1} = \left[\frac{\Delta H_m - \Delta H_{cc}}{\Delta H_{PLA}}\right] \times 100 \quad (1)$$

$$X_{c2} = \frac{\Delta H_m}{\Delta H_{PLA}} \times 100 \quad (2)$$

The thermal stability of all foam samples was determined by thermogravimetric analysis (NETZSCH® TGA/DSC STA 449 F3 Jupiter). Samples were heated at a rate of 5 °C/min from 30 °C to 500 °C under nitrogen atmosphere.

Freeze-fractured foam surfaces were investigated using the scanning electron microscope (FEI® Quanta 400). Pore size was measured from SEM micrographs and approximately 150 to 200 pores were investigated for each specimen. As pores were oval only the largest diameter was determined.

2.4 Physical and mechanical properties tests

Foam density was measured by the buoyancy method described in ASTM standard D792. Three specimens of every sample were measured and the average value was reported. Specimen width and length were 25 mm, and specimen thickness was 2.5 to 3.0 mm. Specimens were weighed on an analytical balance in air and in n-hexane with a density of 0.6954 g/cm^3. Void fraction (VF) was calculated from equation (3), according to Luo-Jiang *et al*. (2013), where ρ_1 and ρ_2 are the density of foam and unfoamed PLA samples, respectively:

$$\%VF = 1 - \left(\frac{\rho_1}{\rho_2}\right) \times \quad 100 \quad (3)$$

The Izod impact strength was determined using Zwick® 5102 apparatus in accordance with ASTM standard

256. The tensile properties were analyzed in accordance with ASTM standard D412 die C. The specimens were tested at room temperature using LLOYD® LR10K equipment. The crosshead speed was 5 mm/min. At least six specimens were tested from every sample. An average value with standard deviations was reported.

2.5 *In-vitro* degradation test

The *in-vitro* degradation of foam samples was evaluated by adding a dry foam sample (1 x 1 cm) to 500 µl of lysozyme solution (4 mg of lysozyme in 1 mL of phosphate buffer solution (PBS) with pH 7.4). The sample was then incubated in the solution at 37°C for 8 weeks (Zhang & Cui, 2012). The lysozyme solution was replaced every week with fresh solution. At determined time intervals, the samples were removed from the solution, washed with distilled water and dried in a freeze dryer. The % weight loss was calculated from equation (4) (Rakmae, Ruksakulpiwat, Sutapun, & Suppakarn, 2012), where W_0 was the initial weight and W_d was the dried weight after degradation:

$$\%Weight\ loss = \left[\frac{W_0 - W_d}{W_0}\right] \times 100 \quad (4)$$

2.6 Cytotoxicity test

MG-63 cells, osteoblast-like cells, were cultured in alpha-MEM medium)Gibco™, Invitrogen, Carlsbad, CA, USA(with the addition of 1% penicillin/ streptomycin, 0.1% fungizone, and 10% fetal bovine serum. Incubation took place at 37 °C in a fully humidified atmosphere at 5% CO_2. MG-63 cells)5 x 10^4 cells(were seeded onto the surface of foam samples. The surface of the foam samples was polished using sandpaper before using in this step. The medium was changed every three days during culture. Osteogenic medium)OS(was used for osteoblast differentiation of the MG-63 cells (Sangkert, Kamonmattayakul, Chai, & Meesane, 2016). MG-63 cells were cultured in a culture plate for 1, 3, 5 and 7 days. MG-63 cell proliferation was measured by WST-1 assay and cell proliferation was quantified according to the manufacturer's instructions (Hsueh, Savitha, Sadhasivam, Lin, & Shieh, 2014). The absorbance of each sample was recorded at 420 nm using a Multiskan™GO Microplate Spectrophotometer.

The morphology of cells that adhered to the foam sample was investigated by scanning electron microscope (FEI® Quanta 400). The seeded foam samples were washed with PBS and fixed with 10% neutral formalin buffer for 5 h at 4 °C. The samples were dried in a freeze dryer and coated with gold prior to investigation.

2.7 Statistical analysis

One-way analysis of variance (one-way ANOVA) was performed using SPSS software (version 20.0) at 0.05 significance level ($p<0.05$). Significant differences among samples were analyzed using Tukey's honestly significant difference (HSD) test. When the average values were significantly different at $p<0.05$, samples were identified by letters, e.g. A, B, C, D, AB and CD.

3. Results and Discussion

3.1 Foam characterization

In the DSC thermograms of samples obtained from the first heating scan, all foams showed a cold crystallization temperature (T_{cc}), attributed to the double melting peak typical of PLA and other polyesters (Figure 1). Transition temperatures (T_g, T_{cc} and T_m) and degrees of crystallinity are listed in Table 2. PEG acted as an effective plasticizer of PLA because the T_g of PLA significantly decreased after the addition of ~ 5 wt% of PEG to the blend. T_g of PLA was ~ 64°C whereas T_g of 0C5P was ~ 57 °C. Chitosan promoted the plasticization effect of PEG because T_g of samples decreased with increasing chitosan content. This synergy was clearly observed in the 3C5P sample, which showed the lowest T_g, i.e. 55 and 50°C for unfoamed and foam sample, respectively. Both PEG and chitosan acted as nucleating agents by reducing T_{cc} of PLA. As shown in Table 2 (unfoamed samples), PLA did not show T_{cc} while 3C0P, 0C5P and 3C5P showed T_{cc} at 104, 100 and 95°C, respectively. It seemed that a mixture of PEG and chitosan produced the lowest T_{cc}. T_{cc} of PLA foam sample was 101°C and it significantly dropped to 82°C after mixing with chitosan and PEG (3C5P foam sample). The occurrence of cold crystallization affected the melting enthalpy of PLA. In this case, ΔH_m in the DSC thermogram was a result of the initial crystallinity of PLA and the crystallinity during heating in DSC analysis (ΔH_{cc}). By excluding ΔH_{cc}, X_{c1} represented the original degree of crystallization of samples. Without PEG (1C0P and 3C0P), chitosan did not affect the degree of crystallinity (X_{c1}) of PLA foam sample. PEG increased the

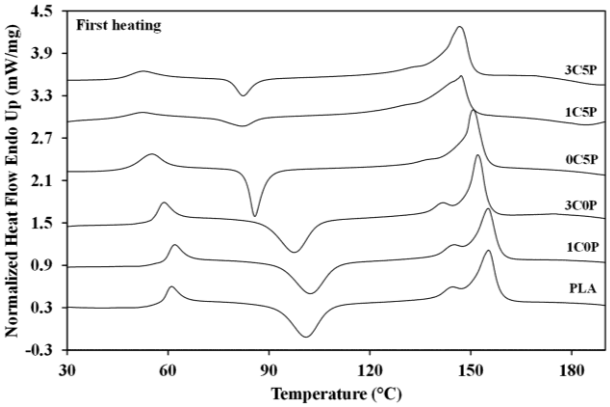

Figure 1. DSC thermograms of samples.

Table 2. Thermal properties of samples.

Foam sample	T_g (°C)	T_{cc} (°C)	T_{m1} (°C)	T_{m2} (°C)	X_{c1} (%)	X_{c2} (%)
PLA	59.6	101.0	144.8	155.3	9	43
1C0P	60.5	102.4	145.2	155.2	8	42
3C0P	57.4	97.6	141.9	152.1	7	41
0C5P	51.8	85.8	138.6	150.8	24	44
1C5P	50.3	82.4	132.4	147.2	44	49
3C5P	49.7	82.0	134.3	146.7	36	44

degree of crystallinity (X_{c1}) of PLA foam sample. However, PEG and chitosan produced only slight changes to the overall degree of crystallinity (X_{c2}) of samples.

SEM micrographs of samples show closed-cell morphologies with oval pores (Figure 2). This cellular structure was also found in polyethylene foam (Almeida, Demori, Zattera, & Zeni, 2007; Sun-Qi et al., 2018) and PLA foam (Zimmermann *et al*., 2013) prepared by compression molding using AZDC. The addition of chitosan and PEG decreased pore size and pore size distribution (Table 3) because chitosan and PEG acted as a nucleating agent for heterogeneous foam nucleation. Our results support previous findings that chitosan and PEG acted as a nucleating agent for foam nucleation. In polystyrene and polypropylene foams prepared by extrusion with AZDC as a blowing agent, chitosan acted as a nucleating agent by reducing the size of cells (Vázquez-González-Núñez *et al*., 2009). Chen-Turng *et al*. (2015) found that the addition of 10 wt% of PEG decreased the pore size in a PLA scaffold.

Foam density was strongly influenced by void fraction (%VF). The foams with higher void fractions had lower densities (Table 3). There was no correlation between foam density and pore size. It seemed that foam density was related to the degree of crystallinity, except in the 3C0P sample. Thermal degradation temperatures of samples are listed in Table 3 in terms of $T_{d\ onset}$ and T_d. Obtained from DTG curves from thermogravimetric analysis, the onset temperature ($T_{d\ onset}$) was the temperature at the initial weight loss and T_d was the temperature at the peak of a DTG curve. The addition of chitosan and PEG slightly affected the thermal stability of PLA.

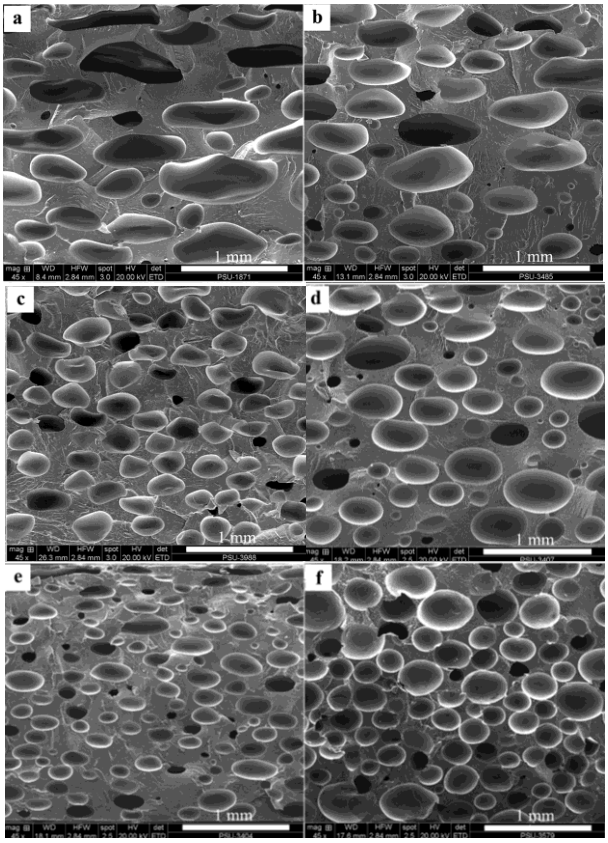

Figure 2. SEM micrographs of samples: (a) PLA, (b) 1C0P, (c) 3C0P, (d) 0C5P, (e) 1C5P, and (f) 3C5P.

Table 3. Physical properties and average pore diameters of samples.

Foam sample	Density (g/cm³)	VF (%)	Average pore diameter (μm)	Degradation temperature (°C)	
				$T_{d\ onset}$	T_d
PLA	0.746 ± 0.007^{B}	39.0 ± 0.5	495 ± 200	332	356
1C0P	0.723 ± 0.002^{C}	40.9 ± 0.2	450 ± 175	326	354
3C0P	0.819 ± 0.009^{A}	33.0 ± 0.7	316 ± 60	327	356
0C5P	0.760 ± 0.003^{B}	37.8 ± 0.2	355 ± 157	314	340
1C5P	0.816 ± 0.004^{A}	33.3 ± 0.3	282 ± 107	326	354
3C5P	0.759 ± 0.014^{B}	37.9 ± 1.1	300 ± 77	323	354

Footnotes: Average values of foam density with different letters in the same column are significantly different at $p<0.05$.

3.2 Mechanical properties

The purpose of using chitosan in the present study was to evaluate the putative bioactivity of chitosan in PLA foam for tissue regeneration. We did not expect reinforcing behavior from chitosan. The addition of chitosan reduced the Izod impact strength and tensile properties of PLA (Table 4). The incompatibility between PLA and chitosan might have been due to the relatively large particle size of the chitosan (~ 149 μm). Reduced tensile properties were previously reported for PLA/chitosan blend (Râpă-Vasile *et al*., 2016). Likewise, chitosan decreased the tensile strength of LLDPE (Isa & Mohammed, 2015). The plasticized PLA without chitosan (0C5P) showed increased Izod impact strength and was the toughest of all the samples tested. The impact strength of the plasticized PLA foams decreased with increasing chitosan content (3C5P<1C5P<0C5P). All plasticized PLA foams with and without chitosan showed poorer tensile properties than PLA foams. Impact strength might be more affected by chitosan than by the degree of crystallinity, foam density or pore size. Notably, 1C5P, having higher density, the highest degree of crystallinity and the lowest average pore size, showed lower impact strength, modulus and tensile strength than 0C5P. The data presented in Figure 3 confirmed that, as with impact strength, density did not have a significant effect on the modulus and tensile strength.

3.3 *In-vitro* degradation

Data of the %weight loss of samples after incubation were statistically analyzed and the average values were significantly different at $p<0.05$. PLA was used as a control sample. The weight loss of all samples increased with increasing incubation time and PLA showed the highest weight loss (Figure 4). At the end of the 2nd week, PLA showed a 5% weight loss while the weight loss of the other samples ranged from 0.5% to 1.3%. After two weeks, the weight loss of PLA was almost constant, whereas the other samples showed a constant weight loss from the 6th week. At the end of the experiment, samples could be ranked by weight loss in the following order: 3C0P<0C5P<1C0P<3C5P<1C5P <PLA. PLA showed a 5.7% weight loss while the weight lost

Table 4. Impact strength and tensile properties of samples.

Foam sample	Izod impact strength (kJ/m^2)		Tensile properties		
	Unnotched	Notched	E (MPa)	σ_b (MPa)	ε_b (%)
PLA	4.9 ± 1.0^{B}	1.6 ± 0.2^{AB}	366 ± 20^{A}	18.4 ± 2.1^{A}	8.3 ± 1.3^{B}
1C0P	3.4 ± 0.4^{C}	1.3 ± 0.3^{B}	216 ± 12DE	7.5 ± 1.4^{C}	5.6 ± 1.2^{CD}
3C0P	4.8 ± 0.5^{B}	1.5 ± 0.1^{AB}	275 ± 40^{BC}	10.8 ± 1.8^{B}	6.6 ± 1.6^{BC}
0C5P	7.3 ± 0.8^{A}	2.0 ± 0.3^{A}	319 ± 30^{B}	16.6 ± 0.6^{A}	7.8 ± 0.5^{BC}
1C5P	5.0 ± 1.0^{B}	1.7 ± 0.4^{AB}	258 ± 27^{CD}	10.6 ± 0.5^{B}	10.7 ± 2.1^{A}
3C5P	3.7 ± 0.8^{BC}	1.5 ± 0.3^{AB}	194 ± 24^{E}	5.0 ± 0.8^{D}	3.7 ± 0.5^{D}

Note: Average values with different letters in the same column are significantly different at $p<0.05$.

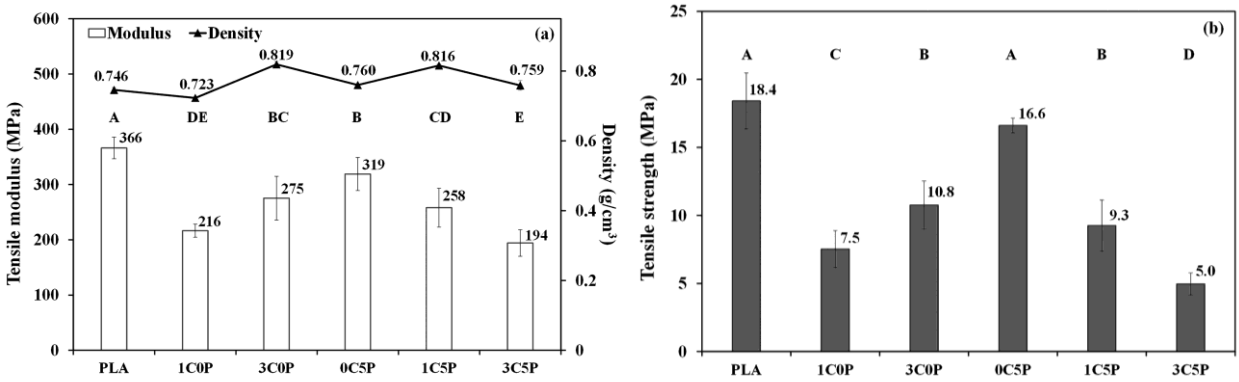

Figure 3. Tensile properties of samples: (a) tensile modulus and (b) tensile strength, average values with different letters are significantly different at $p<0.05$.

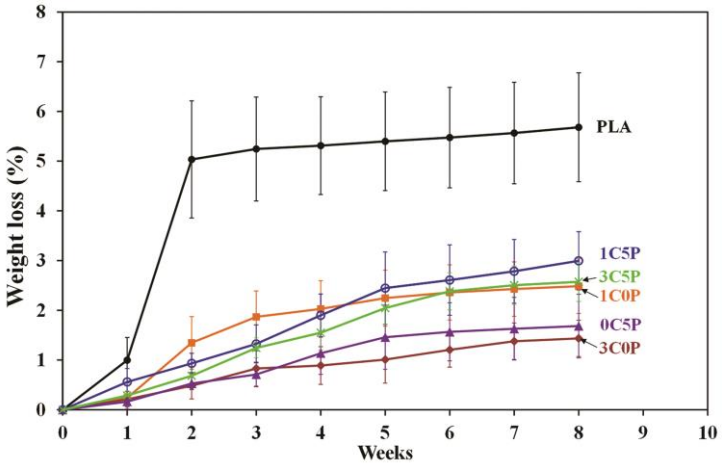

Figure 4. Weight loss as a function of incubation time of samples.

by of the other samples ranged from 1.4% to 3.0%. The addition of chitosan to PLA significantly reduced weight loss. Chitosan reduced %weight loss by neutralizing the acid product of the degradation of PLA and restraining self-catalysis in the PLA degradation process (Li, Ding, & Zhou, 2004). Paradoxically, 0C5P showed relatively little weight loss. This sample will be investigated more deeply in future study.

3.4 Cytotoxicity

The WST-1 assay used in the cytotoxicity evaluation is a colorimetric assay using tetrazolium salt (WST-1) for the quantitation of viable cells. The occurrence of formazan dye from the cleavage of WST-1 is related to the number of viable cells in the cell culture and can be detected by absorption at suitable wavelengths. Increased absorbance is the result of an increase in the number of viable cells present in the culture (Hsueh *et al*., 2014). The data were statistically

analyzed in a similar way to the data from the *in-vitro* degradation test. The proliferation of MG-63 cells increased with cell culture time (Figure 5). This result demonstrated the non-cytotoxicity of all the foam samples. The addition of 1 wt% chitosan (1C0P) slightly changed cell proliferation on PLA whereas 3 wt% chitosan (3C0P) reduced proliferation more significantly. The addition of PEG (0C5P) did not inhibit cell proliferation on PLA. The addition of chitosan in conjunction with PEG inhibited cell proliferation on PLA. This result was similar to the result from the *in-vitro* degradation test. MG-63 cells on the surface of samples are shown in SEM micrographs (Figure 6). The white arrows indicate areas covered by spreading cells (Balu, Sampath Kumar, Ramalingam, & Ramakrishna, 2011). Cell adhesion is visible in the white circles. The appearance of cells on the surfaces of all samples verified the non-cytotoxic reactivity of the samples. Scaffold for bone tissue engineering requires relatively low weight loss and high cell proliferation. In the present study, 1C0P and 0C5P were better in regard to these properties than PLA.

4. Conclusions

PLA foam with and without chitosan and/or poly(ethylene glycol) was successfully prepared by compression molding using azodicarbonamide as a chemical blowing agent. Chitosan was used as a bioactive component and poly(ethylene glycol) was used as a plasticizer. The PLA foam samples containing chitosan and poly(ethylene glycol) showed the lowest T_g, T_{cc} and T_m and contributed the highest

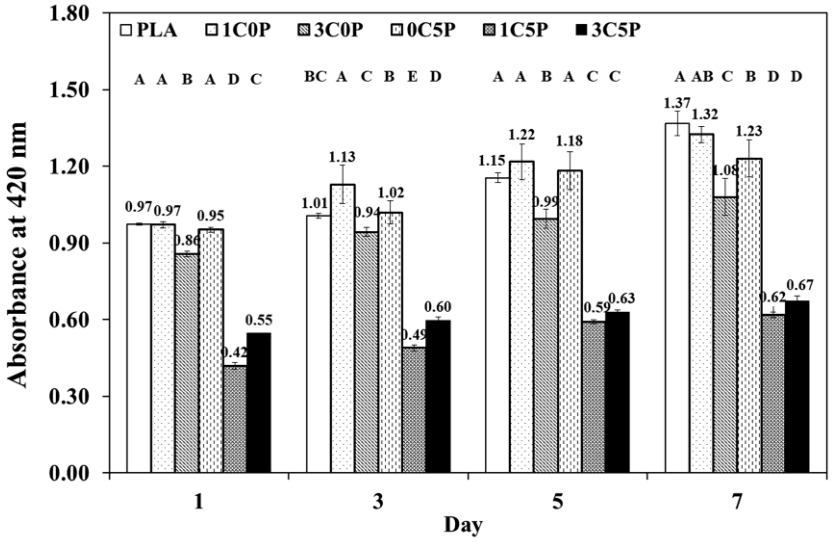

Figure 5. MG-63 cell proliferation on the polished surface of samples at 1, 3, 5 and 7 days, average values with different letters on the same day are significantly different at $p<0.05$.

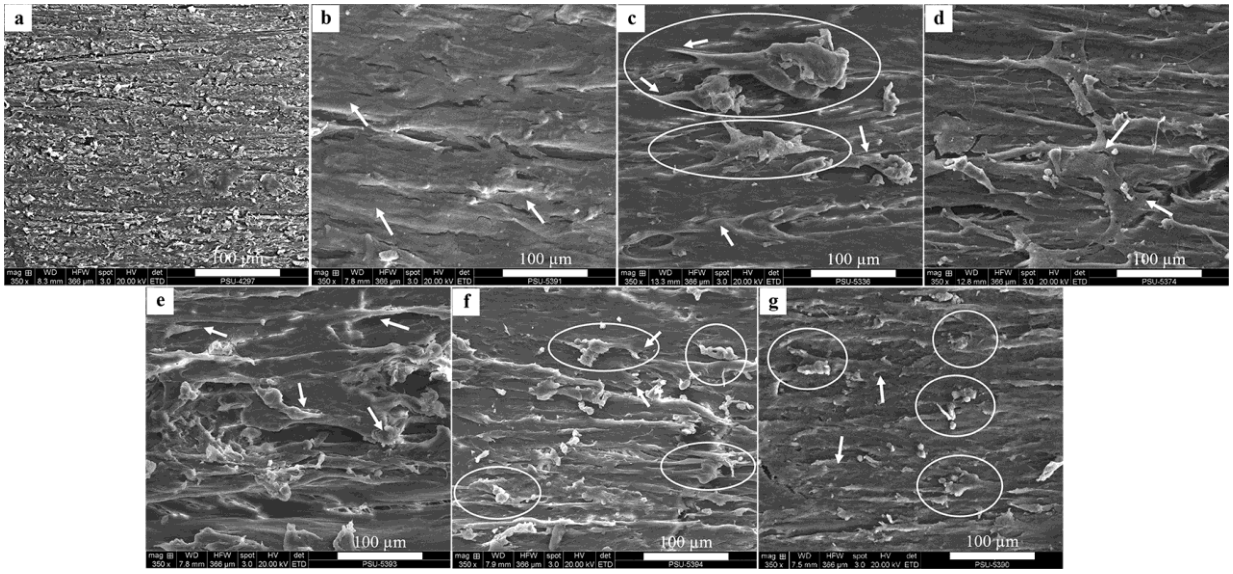

Figure 6. SEM micrographs of cell adhesion on the polished surface of samples after 7 days of cell culture: (a) PLA without cell culture (b) PLA, (c) 1C0P, (d) 3C0P, (e) 0C5P, (f) 1C5P, and (g) 3C5P.

ΔH_{cc}. A closed cell-morphology was obtained, which is common in plastic foam prepared with AZDC in the melt condition. Chitosan and poly(ethylene glycol) decreased pore size and the pore size distribution of PLA foam. The foam samples 0C5P and 1C5P showed higher impact strength than PLA while the other samples showed lower impact strength. The addition of chitosan and poly(ethylene glycol) impaired the tensile properties of PLA. In the cytotoxicity test, cell proliferation on samples 1C0P and 0C5P, though slightly lower than on PLA, indicated their potential for tissue engineering scaffold application. But in the *in-vitro* degradation test, both samples showed much lower weight loss than PLA. The present study demonstrated the positive effect of chitosan and poly(ethylene glycol) on PLA foam for tissue engineering scaffold application. However, this work was the preliminary work and more studies need to be done to obtain 3D interconnecting pore structure in PLA foam by using AZDC and shaping in the compression molding.

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