

Preparation of elastic chitosan/poly(L-lactide-co- ϵ -caprolactone) macroporous scaffolds by acetylation and particulate leaching

Jim Ik Lim¹ · Yun-Cheol Na² · Woo-Kul Lee¹

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Abstract For soft tissue engineering applications, 3-D macroporous acetylated chitosan/poly(L-lactide-co- ϵ -caprolactone) (PLCL) scaffolds were prepared by acetylation and particulate leaching using sodium acetate in an acidic water/dioxane solution. Acetylated 5 wt% chitosan/PLCL scaffold of 90% porosity was determined and confirmed through various tests. The physiochemical properties of acetylated chitosan/PLCL hybrid scaffolds were examined by measuring water contact angles, pore morphology and interconnectivity using scanning electron microscopy (SEM), and dye release testing. In addition, mechanical properties such as tensile strength and bending stress recovery for determining the elasticity of scaffolds were measured. The fibroblast cell line NIH-3T3 was used to test relative cell affinities for the acetylated chitosan/PLCL vs. normal chitosan/PLCL films and porous scaffolds. The acetylated chitosan/PLCL films and scaffolds showed a high initial cell adhesion after 4 h of cell culture and increased cell proliferation compared to that of the control. The acetylated chitosan/PLCL scaffolds produced by particulate leaching showed a highly porous structure and improved the biocompatibility and stability of chitosan compared to that of chitosan-coated PLCL scaffolds. Thus, these scaffolds may be very useful for a variety of tissue engineering applications.

Keywords Chitosan scaffold · Porous structure · Particulate leaching · Acetylation · Elastic PLCL

1 Introduction

Recently, much research has focused on tissue engineering approaches involving the transplantation of cells grown in culture onto biodegradable scaffolds that behave as native, tissue-like materials for cell attachment [1, 2]. Biodegradable materials have important applications in the field of tissue engineering and synthetic biodegradable polymers have been developed for skin or bone tissue engineering e.g., poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA), poly(ϵ -caprolactone) (PCL), poly(glycolide-co-caprolactone) (PGCL) and poly(L-lactide-co- ϵ -caprolactone) (PLCL) [3–7]. However, limitations of these synthetic polymers, such as low cell affinity and irregular hydrophilic ratios, have also been reported [8]. To overcome these disadvantages, various modifications of these materials, such as collagen coated polymers, have been prepared and studied. The immobilization of natural molecules by chemical modification and plasma surface treatments has also been studied [9]. However, the low stability of the modified polymers and the difficulty associated with their quantitative analysis remain unresolved problems [10].

More recently, 3-D structured chitosan/poly (L-lactide) (PLLA) has been reported to show improved properties of the hybrid polymer surface i.e., high biocompatibility, low cost, and enhanced mechanical properties [11–15]. Chitosan (derived from chitin) is a naturally derived crystalline polysaccharide. It has gained much attention as a biomaterial in diverse tissue engineering applications owing to its low cost, large-scale availability, antimicrobial activity,

✉ Woo-Kul Lee
leewo@dankook.ac.kr

¹ Laboratory of Biointerfaces/Tissue Engineering, Department of Chemical Engineering, Institute of Tissue Regeneration Engineering, College of Engineering, Dankook University, Jukjeon-dong, Yongin-si, Gyeonggi-do, Republic of Korea

² Joint Bio-analytical Research Team, Korea Basic Science Institute, Seoul, Republic of Korea

minimal foreign body reaction, and biocompatibility [16]. Consequently, various types of chitosan-based materials, including acetylated chitosans and chitosans hybridized with synthetic polymer, have been tested in tissue regeneration [11, 17, 18].

The advantages of the salt-leaching method for the fabrication of 3-D porous structures are that the pore size can be controlled and the microstructural morphology is easier and quicker to produce [2, 19]. Chitosan is normally insoluble at a $\text{pH} < 7$. However, in a dilute acid solution ($\text{pH} < 6$), the free amino groups on the chitosan skeleton are protonated, resulting in dissolution. A 3-D porous chitosan scaffold was previously prepared by the conventional freeze-drying method but, owing to its restrictive microstructural morphology, this scaffold was not ideal for tissue engineering because of its low mechanical strength and poor cell affinity [20–22]. In the case of salt leaching of chitosan in an acidic solution, the degree of ionization of the salt is very important for the preparation of scaffolds of various morphologies with improved properties. A salt with low ionization under acidic conditions is required. In this study, sodium acetate was used as the porogen because it is a readily obtainable salt with a relatively low pK_a (acetic acid=4.76) and chitosan is soluble in acetic acid solution.

In this study, in order to enhance the mechanical strength and flexibility of chitosan, biodegradable poly(L-lactide-co- ϵ -caprolactone) (PLCL) (50:50) copolymers were chosen. These copolymers were utilized because they exhibit different elastic recoveries as amorphous or highly crystalline polymers depending on the lactide/caprolactone molar ratio, as reported in a previous study [6].

The objectives of this study were to (1) design a novel chitosan/poly(L-lactide-co- ϵ -caprolactone) (PLCL) hybrid scaffold by using sodium acetate particulate leaching and acetylation, (2) evaluate the influence of acetylated chitosan on the acetylated chitosan/PLCL scaffolds, and (3) determine the optimal concentration of chitosan/PLCL to generate a highly porous, biocompatible structure.

2 Materials and methods

In this study, 86% deacetylated chitosan (Korea-Chitosan Co, Seoul, Korea), approximate molecular weight 1000 kDa was used for the scaffold. PLCL (molar ratio 50:50, number average molecular weight (M_n): 2.2×10^5) was polymerized and purified using the methods described in a previous report [23]. Absolute ethanol, acid blue-25, caprolactone, sodium acetate, acetic anhydride, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade. Ultrapure water from a Milli-Q water purification system was used to prepare all aqueous solutions.

A PLCL/particulate mixture [10:90 (w/w)] was obtained by dissolving PLCL [10% (w/v)] in dioxane and adding 90 wt% sodium acetate (100–250 μm). A mixture of chitosan and particulate [10:90 (w/w)] was prepared by dissolving chitosan [3% (w/v)] in 2% acetic acid and adding 90 wt% sodium acetate (100–250 μm). Chitosan/PLCL/particulate mixtures (chitosan in PLCL:5, and 10 wt%) were obtained by mixing the PLCL/particulate and chitosan/particulate mixtures. The chitosan/PLCL/particulate mixtures were then injected into molds ($20 \times 20 \times 5 \text{ mm}^3$), frozen at -70°C for 24 h, and then lyophilized for 24 h. The lyophilized chitosan/PLCL/particulate materials were gradually rehydrated by serial washing in graded ethanol–water mixtures (100, 90, 80, 70, and 50% v/v), for 2 h at each stage. At the end of the final wash, any undissolved salt was removed from the scaffolds by immersion in deionized water at 25°C for 72 h, freezing at -70°C for 24 h, followed by lyophilization for 24 h. The lyophilized porous scaffolds were immersed in an acetylation solution (acetic anhydride:ethanol:Tween 20 solution, 20:10:3 v/v/v). Tween 20 was used to improve contact between chitosan and acetic anhydride for reaction. The reaction was stopped after 50 h by adding 5% NaOH solution. The hydrogels were immersed in 95% EtOH solution for one day to remove any remaining acetic anhydride and then washed with distilled water and freeze-dried. As a control, a normal chitosan/PLCL porous scaffold without the acetylation step was prepared by the same method.

Chitosan/PLCL mixtures without salt were prepared by the same method as the scaffolds and coated (200 μl per slide) onto glass slides ($20 \times 20 \text{ mm}^2$). After 3 weeks at room temperature, the coated slides were immersed in the acetylation solution. The reaction was terminated after 50 h by adding 5 wt% NaOH solution. The films were immersed in a 95% EtOH solution for one day to remove any remaining acetic anhydride, washed with distilled water, and then dried at room temperature for 3 days. The following control films were prepared: a normal chitosan/PLCL film without the acetylation step, chitosan coated on PLCL, and pure PLCL films were prepared by similar methods.

In order to study the morphology of the acetylated chitosan/PLCL porous scaffolds, the interconnectivity of the pores was observed in cross sections using a field-emission scanning electron microscope (FE-SEM:S-4700; Hitachi, Tokyo, Japan).

The stability of acetylated chitosan on the acetylated chitosan/PLCL (5 and 10 wt%) and acetylated chitosan-coated PLCL films was determined following mechanical stress caused by rotation of the films in water, (80 rpm in distilled water; SH-802F incubator, Human Corp., Seoul, Korea) applied for 0, 4, 24, 48, and 72 h. The water contact angle of the dried film was measured using a contact angle meter (Phoenix 150; Surface Electro Optics, Seoul, Korea).

The tensile strength of films was measured using an INSTRON universal testing machine (model 5567, carton, MA, USA) employing a gauge length of 10 mm and a 5-N maximum load cell with a crosshead speed of 1 mm/min.

The recovery from repetitive bending stress of acetylated chitosan/PLCL films ($30 \times 10 \times 1 \text{ mm}^3$) was tested using a universal testing machine. A 5-N load cell with a crosshead speed of 1 mm/min (bending angle 180°) was used for this purpose. The recovery was calculated as Recovery (%) = $A1/A0 \times 100$, where A0 indicates the original angle and A1 indicates the final angle after stress release.

A solution of the dye, acid blue-25 (1 mg /ml) in distilled water and ethanol (50:50) mixture was prepared and 5 wt% chitosan/PLCL or acetylated 5 wt% chitosan/PLCL scaffolds ($20 \times 20 \times 5 \text{ mm}^3$) were submerged for 12 h under vacuum conditions. Each scaffold was subsequently suspended in 10 ml PBS and incubated at 37°C with rotation at 30 rpm for 0, 0.5, 1, 2, and 3 h. After incubation, the releasate, concentrated by lyophilization, was collected and added to a 96-well plate. Absorbance at 600 nm was measured to quantify the released dye using a micro plate reader.

For the initial cell adhesion tests, the acetylated chitosan/PLCL films were pre-wetted with cell culture medium (Dulbecco’s modified Eagle’s medium with 2 mM L-glutamine and 10% fetal bovine serum) and incubated at 37°C in a 5% CO_2 atmosphere for 12 h. After incubation, the medium was aspirated, and a 100- μl suspension of NIH-3T3 cells (1×10^4 cells) in culture medium was added directly to each film ($20 \times 20 \text{ mm}^2$) in a culture dish well. After a 1-h incubation, 900 μl of medium was slowly added to each well. The acetylated chitosan/PLCL scaffolds ($20 \times 20 \times 5 \text{ mm}^3$) were similarly pre-wetted with culture medium and incubated at 37°C with 5% CO_2 as described for the films. After 12 h, the medium was aspirated, and NIH-3T3 cells were added (3×10^5 cells in 1 ml of medium) directly to each scaffold and incubated for another 1 h, after which 2 ml of medium was slowly added to each well. The initial adhesion and proliferation properties were determined by using the MTT assay. NIH-3T3 cells were obtained from the ATCC, Manassas, VA, USA.

3 Results and discussion

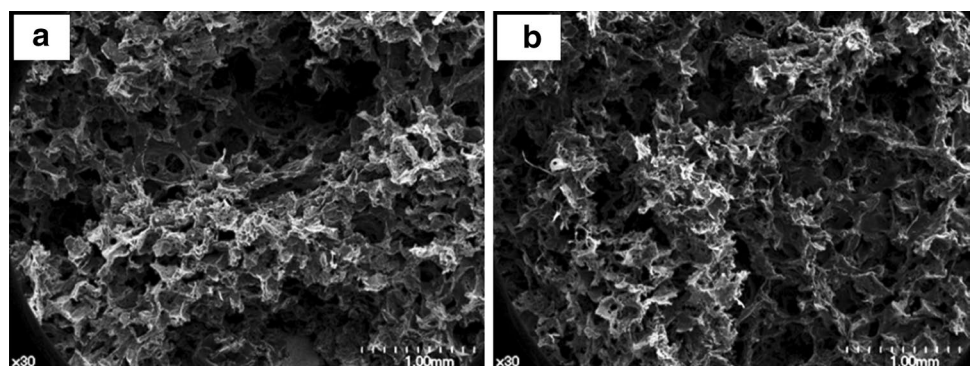
In the particulate-leached scaffolds, no aggregation of chitosan or closing of pores owing to salting-out was observed. As indicated in Fig. 1, porosity and interconnectivity increased to some degree with increase in the proportion of chitosan (Fig. 1a, b). Moreover, the scaffolds prepared with sodium acetate showed round shaped pores with homogeneous pore structure despite the angular crystalline solid form of the salt. Pore structure is important for cell affinity and growth rate in tissue engineering applications. A round shaped pore structure was recently reported as optimal for tissue regeneration [24]. We suggested that the round shape pore structure forms as a result of slight dissolution of the salt surface under acidic aqueous solution conditions.

The stability of the acetylated chitosan on the acetylated chitosan/PLCL and acetylated chitosan-coated PLCL films was evaluated by measuring the water contact angle following the application of mechanical stress for 72 h in distilled water. After 24 h, the water contact angle of the acetylated chitosan-coated PLCL films was found to be almost the same as that of pure PLCL films (Table 1). However, water contact angle measurements revealed that acetylated chitosan remained stable when the acetylated chitosan/PLCL films (5 and 10 wt%) were placed under mechanical stress. Furthermore, the water

Table 1 Correlation of between mechanical stress and water contact angle (θ) of acetylated chitosan/PLCL and acetylated chitosan-coated PLCL films

Films	Time (h)				
	0 ($^\circ$)	4 ($^\circ$)	24 ($^\circ$)	48 ($^\circ$)	72 ($^\circ$)
Acetylated chitosan/PLCL 5 wt%	72	70	68	69	68
Acetylated chitosan/PLCL 10 wt%	69	70	68	68	67
Acetylated chitosan-coated PLCL	64	63	75	76	75
Pure PLCL	78	77	75	75	74

Fig. 1 SEM images of acetylated chitosan/PLCL scaffolds with chitosan concentrations of **a** 5%, **b** 10% (cross-section, $30\times$)



contact angle of the 5 wt% acetylated chitosan/PLCL films showed similar trend with 10 wt% acetylated chitosan/PLCL films.

The recovery of the acetylated chitosan/PLCL films in response to repetitive bending stress was compared with that of pure acetylated chitosan film, which was used as the control. The recovery of acetylated chitosan/PLCL films increased with increasing PLCL concentration. In the case of pure acetylated chitosan films, the films were broken after 5 flexes (Table 2). Acetylated 5 wt% chitosan/PLCL film showed the highest recovery efficiency after 30 repetitions ($***P < 0.05$). Therefore, we conclude that the good elastic properties of acetylated chitosan/PLCL film could be applied to 3-D porous materials for regeneration of various soft tissues such as artificial skin and as an anti-infective barrier film after operations.

Figure 2a, b illustrates the deformation behaviors of the pure PLCL film (control) and acetylated chitosan/PLCL film (5 wt% chitosan in PLCL). The tensile strength and Young's modulus of acetylated chitosan/PLCL film were decreased by the addition of chitosan (Fig. 2b). In addition, elongation at break was decreased about 1.5-fold compared with that of pure PLCL films (Fig. 2a). However, acetylated

5 wt% chitosan/PLCL film showed sufficient elongation (200%) for a soft tissue engineering scaffold. These results suggest that 5 wt% acetylated chitosan/PLCL scaffold is suitable for soft tissue engineering applications.

In Fig. 3, the acetylated chitosan/PLCL scaffold showed an about two-fold higher initial dye release rate than the normal chitosan/PLCL scaffold (release time: normal chitosan/PLCL scaffold (%); acetylated chitosan/PLCL scaffold (%), 0.5 h: $7.1 \pm 4/13.7 \pm 3$; 1 h: $13.1 \pm 2/25.7 \pm 3$). After 2–3 h, the difference in the amount of dye released from each scaffold had decreased from about two-fold to 1.3-fold or less (release time: normal chitosan/PLCL scaffold (%)/acetylated chitosan/PLCL scaffold (%), 2 h: $22.5 \pm 2/29.1 \pm 4$; 3 h: $24.2 \pm 4/32.6 \pm 5$). We believe that the low release rate from the normal chitosan/PLCL scaffold was due to electrostatic attraction between the cationic chitosan and the anionic dye. The higher initial release rate from the acetylated chitosan/PLCL scaffold is indicative of faster diffusion through the 3D porous structure with lower electrostatic attraction to the polymer surface.

To measure cell affinity of the acetylated chitosan/PLCL, NIH3T3 cells were cultured on both normal chitosan/PLCL and acetylated chitosan/PLCL films.

Table 2 Recover (%) to repetitive bending stress of acetylated chitosan/PLCL films ($n=5$)

Repetitive test no.	1	5	10	15	20	25	30
Films							
5 wt% acetylated chitosan/PLCL	$99 \pm 1^*$	98 ± 3	99 ± 2	97 ± 3	98 ± 2	97 ± 3	$98 \pm 2^{**}$
10 wt% acetylated chitosan/PLCL	$98 \pm 3^\#$	97 ± 2	95 ± 3	96 ± 2	94 ± 1	93 ± 2	$89 \pm 2^{##}$
Acetylated chitosan	64 ± 4	–	–	–	–	–	–

*, ** $P < 0.05$, #, ## $P > 0.05$

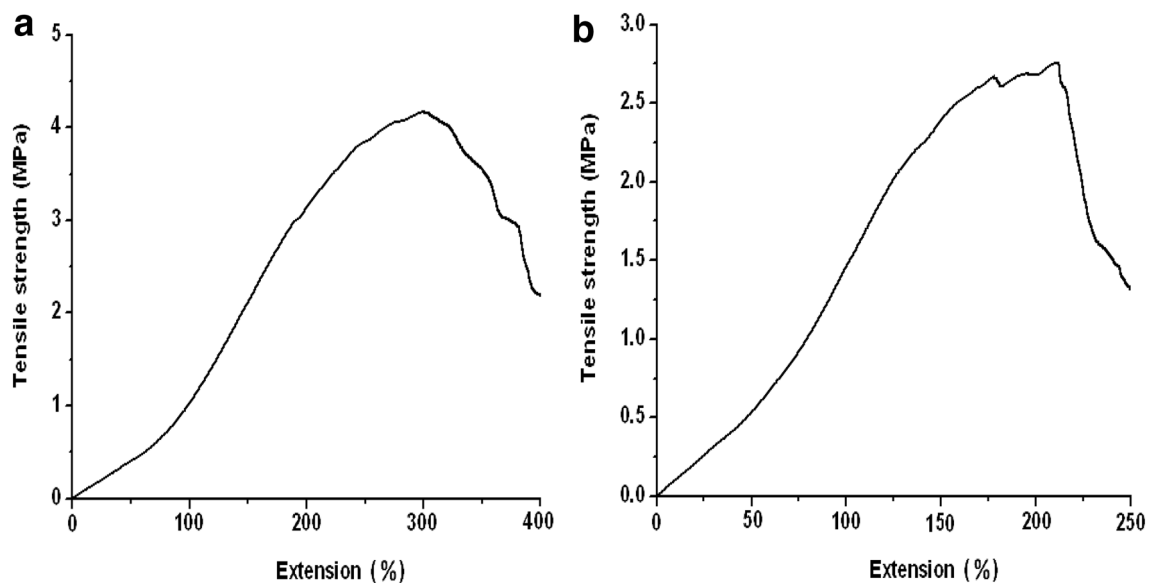


Fig. 2 Tensile strain–stress curve of chitosan/PLCL film (a: PLCL film, b 5 wt% acetylated chitosan/PLCL film)

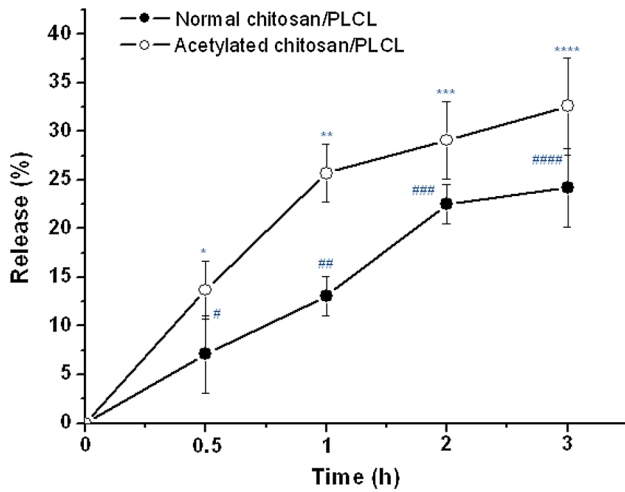


Fig. 3 Acid blue-25 release test from acetylated chitosan/PLCL scaffolds in distilled water and ethanol solution at 37 °C and 30 rpm. ($n=5$, * $^{\#}P<0.05$, ** $^{\#\#}P<0.05$, *** $^{\#\#\#}P<0.05$, **** $^{\#\#\#\#}P<0.05$)

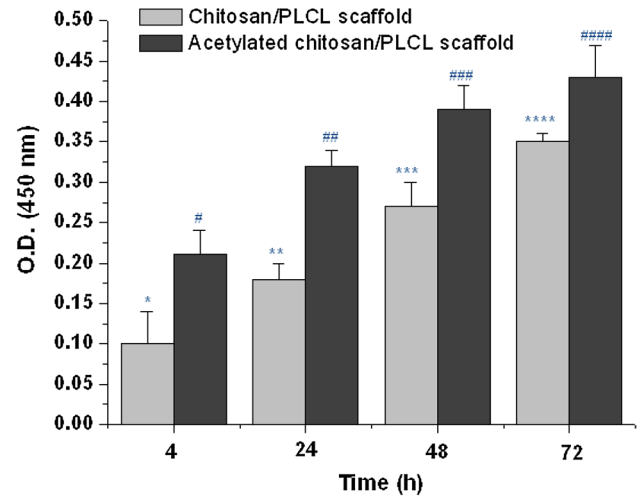


Fig. 5 Difference in cell adhesion and proliferation on chitosan/PLCL scaffolds. ($n=5$, * $^{\#}P<0.05$, ** $^{\#\#}P<0.05$, *** $^{\#\#\#}P<0.05$, **** $^{\#\#\#\#}P<0.05$)

Acetylated film showed highly efficient initial cell adhesion to the films after culturing 4 h. After 48 h, increased cell proliferation on the acetylated films was also observed (Fig. 4). Furthermore, as the cell culture time increased, the extent of the changes in cell adhesion and growth on the acetylated films was higher than that on normal films. In addition, in case of 3-D porous scaffolds, the acetylated chitosan/PLCL promoted highly efficient cell adhesion and growth. Figure 5 shows increased initial cell adhesion efficiency on the acetylated surface. Cell adhesion after 4 h of culture with acetylated scaffold was about two-fold higher than with the normal

chitosan/PLCL scaffold. Moreover, increased cell proliferation was observed after 48 and 72 h of culture. We suggest that initial cell adhesion efficiency and the open pore structure of the scaffold will be very beneficial in tissue engineering. In the optimized scaffolds we have described, the high initial cell adhesion efficiency may be attributed to an open pore structure and acetylated amine groups on chitosan with cell proliferation enhanced by good mass transfer through the open structure.

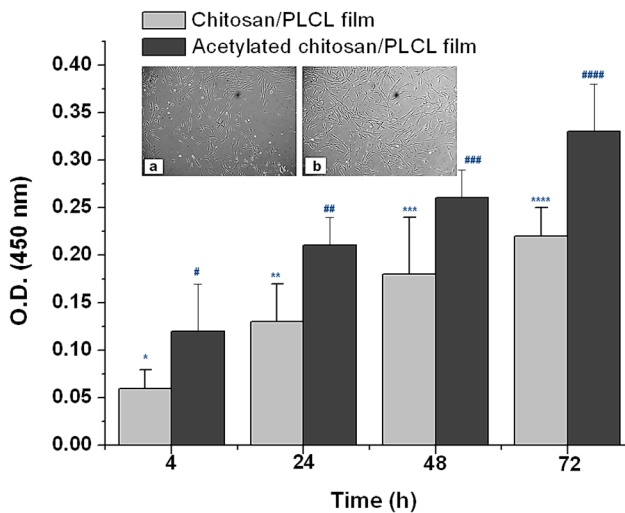


Fig. 4 Difference in cell adhesion and proliferation on chitosan/PLCL films [Microphotography of attached cell after 24 h (a: chitosan/PLCL film; b: acetylated chitosan/PLCL film, $\times 10$)]. ($n=5$, * $^{\#}P<0.05$, ** $^{\#\#}P<0.05$, *** $^{\#\#\#}P<0.05$, **** $^{\#\#\#\#}P<0.05$)

4 Conclusion

3-D porous acetylated chitosan/PLCL scaffolds were prepared by mixing chitosan with elastic biodegradable PLCL (50:50) and using sodium acetate as the porogen in an acidic water/dioxane solution followed by acetylation. Studies of the stability and porous structure of chitosan on the chitosan-coated PLCL and chitosan/PLCL hybrid scaffolds demonstrated improved stability and a highly porous structure in the hybrid scaffolds. Furthermore, cell affinity was improved by acetylated chitosan and the presence of a highly porous structure. Although further studies including further biological evaluations and analysis of the enhanced mechanical properties of the scaffold and assessment of the possible applications of salt leaching to other water-soluble polymers, such as collagen or hyaluronic acid, are required, we believe that the method of preparing elastic acetylated chitosan/PLCL scaffolds presented here may prove useful for regeneration of various tissue types.

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