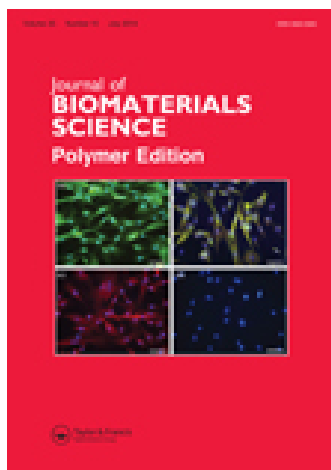


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Fabrication and characterization of Mg/P(LLA-CL)-blended nanofiber scaffold

Heyu Li^a, Tong Wu^a, Yufeng Zheng^b, Hany El-Hamshary^{c,d}, Salem S. Al-Deyab^c and Xiumei Mo^{a*}

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Poly(L-lactic acid-co-ε-caprolactone) (P(LLA-CL)) is a kind of copolymer polymerized from lactic acid and ε-caprolactone. Electrospun P(LLA-CL) nanofibers have good biocompatibility, biodegradability, and mechanical property. However, this type of nanofibers will produce acid groups during the degradation, so that, the pH value of the environment will decrease and result in tissue inflammation. On the other hand, Magnesium (Mg) alloy tissue engineering scaffolds will show alkaline during the degradation because of the electrochemical corrosion. Based on the principle of acid-based neutralization, combination of these two kinds of materials through electrospinning could keep the pH of the degradation environment neutral. In this paper, fabrication and characterization of Mg/P(LLA-CL)-blended nanofiber scaffolds with different ratios will be studied by scanning electron microscopy and universal materials testing machines to observe the morphology and mechanical properties of nanofibers, respectively. Furthermore, PIECs were cultured and seeded on the scaffolds for different time to evaluate the proliferation behavior on the scaffolds by MTT assay. The degradation tests of the samples lasted for three months in phosphate-buffered saline to evaluate the pH values of degradation solutions and the weight loss of nanofibers during degradation. The results showed that the mechanical property and biocompatibility of Mg/P(LLA-CL)-blended nanofibers were worse than that of pure P(LLA-CL). Moreover, the addition of Mg in the nanofibers accelerated the weight loss of the Mg/P(LLA-CL) blending fibers and increased the pH values of the environment during degradation of Mg/P(LLA-CL)-blended nanofibers.

Keywords: electrospinning; P(LLA-CL); Mg; degradation; nanofiber

1. Introduction

Scaffolds, as one of three essential elements, play a pivotal role in the field of tissue engineering, because scaffolds are used for supporting growth of cells, guiding regeneration of tissues, controlling organization of tissues, and releasing growth factors. An ideal scaffold should possess the following characteristics: (i) similar structure and biological functions as the natural extracellular matrix (ECM); (ii) appropriate degradation rate to match the growth of cells and regeneration of tissues; (iii) suitable surface for

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cell attachment and proliferation; and (iv) good mechanical properties to support regeneration of the tissues at the site of implantation.[1–3]

During the past two decades, significant advances have been made in the development of biodegradable polymeric materials. These materials have been good candidates for ideal scaffolds.[3] Currently, a large range of biodegradable polymers, capable of fitting the characteristics of ideal scaffolds are being investigated. They can be categorized as natural materials (such as collagens,[4,5] polysaccharides,[6] inorganic substances, biological derivatives [7]) and synthetic biodegradable materials [8,9] (polyesters,[11] poly amino acids,[12] PEG, and so on). In this study, one synthetic biodegradable material, poly(L-lactic acid-co- ϵ -caprolactone) (P(LLA-CL)), was chosen as one of the experiment materials.

P(LLA-CL), as the copolymer of L-lactic acid and ϵ -caprolactone, has distinctive mechanical property and biocompatibility.[13] However, the lower pH value after degradation restricts its clinical applications.[14] On the other hand, Mg alloy scaffolds possess good mechanical property and biocompatibility for various applications, as well.[15–17] But Mg will show alkaline during the degradation and it is easy to be corroded.[18,19]

Electrospinning, a typical way to prepare nanofibers, was put forward in 1934 by Formhals [20]. Its main principle is that the applied voltage causes a cone-shaped deformation on the drop of polymer solution. Afterwards, a jet will generate from the deformed drop, which jets towards the electrode with higher voltage.[21] Currently, this technology has been regard as an effective method to fabricate nanofibers, composed of a large network of interconnected fibers and pores, which could resemble the morphological structure of ECM.[22,23] That is why electrospinning is one of the most effective methods to fabricate nanofiber scaffolds. In recent years, different polymers have been electrospun into nanofibers for various applications.[24] Most of nanofibers fabricated by electrospinning have been applied in tissue repair and regeneration areas, such as bones,[25,26] blood vessels,[27] nerves,[28] cartilages,[29] and skins.[30] Furthermore, various blending composite nanofibers were fabricated for more applications. For example, electrospun silk fibroin/P(LLA-CL) nanofibers have better mechanical property, biocompatibility, and degradation behaviors than pure P(LLA-CL) nanofibers.[31,32] PU/PCL blending scaffolds satisfies the requirement of blood vessel prosthesis well.[33] Another instance is PAN/Fe₃O₄ nanofiber scaffold, which has good magnetic and mechanical properties.[34] Recently, a kind of Mg/PCL hybrid bone substitute has been fabricated. It was testified that Mg/PCL composites had good biocompatibility, mechanical property, and potential clinical application.[35] Thus, it is obvious that composite nanofibers have more promising prospects.

Therefore, to obtain an ideal type of scaffolds, composite nanofibers of Mg/P(LLA-CL) has been fabricated via electrospinning. Then, the mechanical property, degradation property, and biocompatibility of this composite nanofibers have been tested in comparison with P(LLA-CL) nanofibers.

2. Materials and methods

2.1. Materials

A copolymer of P(LLA-CL) (50:50) ($M_w = 34.5 \times 10^4$ g/mol), which has a composition of 50 mol.% L-lactide, was provided by Gunze Ltd, Japan. Magnesium – 1 wt.% Ca alloy micro-particles (45 μ m) were supplied by Peking University (China).

1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was purchased from Darui Chemical Ltd, China. Phosphate-buffered saline (PBS), sodium azide, penicillin, and thiazolyl blue (MTT) were obtained from Sigma Co. Ltd, USA. Both of the dimethyl sulfoxide (DMSO) and DMEM cultural medium came from Jinuo Biological Medicine Technology Ltd, China. Trypsin and PIECs were bought from Sijiqing Biological Engineering Ltd, China and Cell Bank of Chinese Academy of Sciences, respectively.

2.2. Preparation of the nanofiber scaffolds via electrospinning

Mg/P(LLA-CL) blends (weigh ratios were 1:2, 1:4, and 1:9) and pure P(LLA-CL) were dissolved in HFIP and stirred by magnetic stirrer at room temperature for 12 h. The concentration of all the solutions was 8% g/mL. Then electrospinning solutions were poured into 5.0 mL plastic syringes with blunt-ended needles. Syringes were placed on syringe pumps (789100C, Cole-Pamer, America) and propelled at a rate of 1.0 mL/h. The high voltage power supplies (BGG6-358, BMEI Co. Ltd, China) with the voltage of 16 kV were applied across the needles and ground collectors (aluminum foil), which were located at a distance of 15 cm. After electrospinning, the nanofiber scaffolds were treated in a vacuum drying oven in order to reserve. The parameters of the four groups of nanofiber scaffolds were listed in Table 1.

2.3. Characterization of nanofiber scaffolds

Morphology of the nanofiber scaffolds was observed by scanning electronic microscope (SEM) (JSM-5600, Japan) at an accelerated voltage of 10 kV. The proportions of Mg in nanofiber scaffolds were detected through Energy Dispersive Spectrometer (EDS).

To test the mechanical property of scaffolds, every sample was cut into six pieces of same size (30 mm × 10 mm) at first, then all the samples were located on universal materials tester (H5 K-S, Hounsfield, UK) with a 50 N load cell at the room temperature and the cross-head speed was set as 10 mm/min to stretch the tested nanofibers. Until the samples were broken, the mechanical properties of all the nanofiber scaffolds were obtained.

2.4. Degradation of nanofiber scaffolds

The electrospun nanofiber scaffolds were cut into rectangles (10 mm × 20 mm). Then, 2000 mL of phosphate-buffered solution (pH 7.03 ± 0.10) containing 4.0 mg of sodium azide were prepared as degradation solution. After the degradation solution was sterilized for 2 h in an autoclave, each sample was put into 20 mL of degradation solution. Moreover, all the samples should be sealed up and put on a shaker in 37 °C

Table 1. Parameters of nanofiber scaffold preparation.

Sample	Solute	Mg:P(LLA-CL) ratio (w/w)	Mg content (%)	Solvent
S1	P(LLA-CL)	0	0	HFIP
S2	Mg/P(LLA-CL)	1:9	10	HFIP
S3	Mg/P(LLA-CL)	1:4	20	HFIP
S4	Mg/P(LLA-CL)	1:2	33.3	HFIP

for better simulating the natural environmental conditions *in vitro*. After degradation for 1, 2, 4, 8, and 12 weeks, all the samples were characterized. Morphology of the scaffolds was observed by SEM (JSM-5600, Japan) at an accelerated voltage of 10 kV. Moreover, pH value of degradation solutions was measured by pH-meter for each value averaged from three specimens. In addition, weight loss percentages of all the samples were tested and calculated using gravimetric method.[36]

2.5. Cytocompatibility test

Four groups of electrospun nanofiber scaffolds with glass slides were located in 24-well plates and another group of glass slides without scaffolds was set as control. Then the culture plates were sterilized by alcohol steam for 4 h and PBS solution was used for washing residual alcohol. After being soaked by DMEM, all the culture plates were put in an incubator for 24 h (37 °C, 5% CO₂). After that, 200 μL of PIECs suspension with the cells density of 2.0×10^4 cells/mL was seeded to each well with DMEM (containing 10% FBS) and then incubated in a incubator (37 °C, 5% CO₂). The time points of test were set as 1, 3, and 5 days. At each point, the culture plates were taken out of the incubator. The used DMEM in every well was replaced by 360 μL DMEM and 40 μL MTT solutions. After incubation for 4 h, 400 μL of DMSO was add to each well and the plates were shaken for 30 min at room temperature. Afterwards, solutions in each well were transformed into 96-well plates. Finally, the OD values of the purple solution were measured at 429 nm with a Microplate Reader.

3. Results and discussion

3.1. Morphology of electrospun nanofibers

SEM images of four electrospinning products are shown in Figure 1. It is demonstrated that P(LLA-CL) and Mg/P(LLA-CL) with different Mg content could all be electrospun into nanofibers under the settled conditions successfully. Moreover, the morphologies of the nanofibers are normal though several spherical particles can be seen on the surfaces of the Mg/P(LLA-CL) nanofibers. The appearance of the particles may result from the aggregation of Mg micro-particles. The reason of aggregation may be the incomplete dissolution of Mg micro-particles in electrospinning solutions. As a result, the aggregated Mg micro-particles were jetted on the nanofibers mats.

3.2. Elemental analysis of the electrospun nanofiber scaffolds

EDS elemental analysis pictures of P(LLA-CL) and Mg/P(LLA-CL) nanofibers are shown in Figure 2. From Figure 2(a), it indicates that pure P(LLA-CL) nanofibers consists of Carbon (C) and Oxygen (O), and the trace aluminum (Al) should be the aluminum foil residue. Figure 2(b)–(d) shows that the existence of Magnesium (Mg) in Mg/P(LLA-CL) nanofibers.

Table 2 displays the weight percentages and atomic percentages of Mg in different groups of scaffolds. The results in Table 2 confirm that, with the increase of the proportion of Mg in the electrospinning solutions, the weight percentages and atom percentages of Mg in the nanofiber scaffolds rise as well.

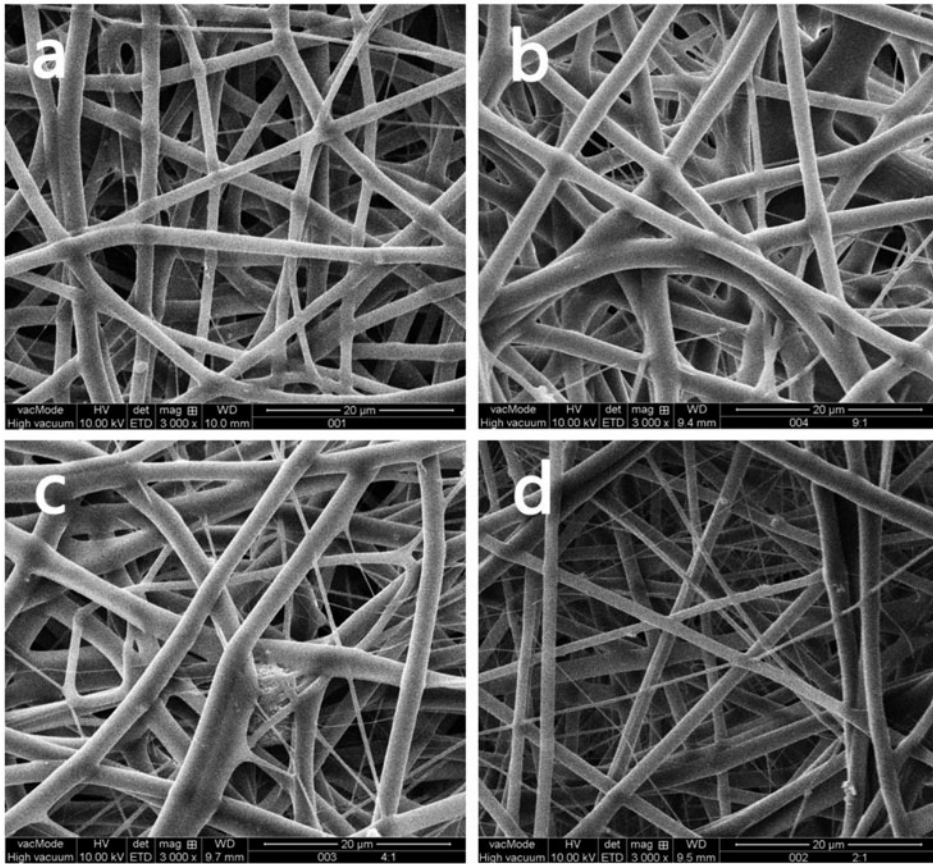


Figure 1. Morphologies of the nanofiber scaffolds. (a) P(LLA-CL); (b) Mg/P(LLA-CL) (1:9); (c) Mg/P(LLA-CL) (1:4); and (d) Mg/P(LLA-CL) (1:2).

3.3. Mechanical property of nanofiber scaffolds

The stress–strain curves of nanofibers are shown in Figure 3. The average tensile strength of P(LLA-CL) nanofibers is about 3.36 MPa and the average elongation at break is about 305.67%. Compared with the pure P(LLA-CL) nanofibers, the average tensile strength of Mg/P(LLA-CL) nanofibers declines, while the average elongation at break of them changes little. The average tensile strength of Mg/P(LLA-CL) (1:9) nanofibers declines to 3.05 MPa, and that of Mg/P(LLA-CL) (1:4) nanofibers drops to 1.15 MPa, whereas that of Mg/P(LLA-CL) (1:2) rises to 2.12 MPa instead of continues declination. This may result from the imperfect mixing of Mg and P(LLA-CL). Mg powders, as metal particles, are difficult to uniformly disperse in the polymers, which might cause the weight imbalance of nanofibers. As a result, the fibers could not resist major tensile force brought from the tester. However, with the increase of Mg amount, the composite nanofibers remain flexible with almost invariable average elongation, which is ascribed to the good elasticity of P(LLA-CL). Furthermore, according to the average tensile strength of Mg/P(LLA-CL) (1:4) and Mg/P(LLA-CL) (1:2) nanofibers, it is estimated that although blending Mg affects mechanical property of Mg/P(LLA-CL) nanofibers, the good mechanical property of Mg itself enhances the average tensile

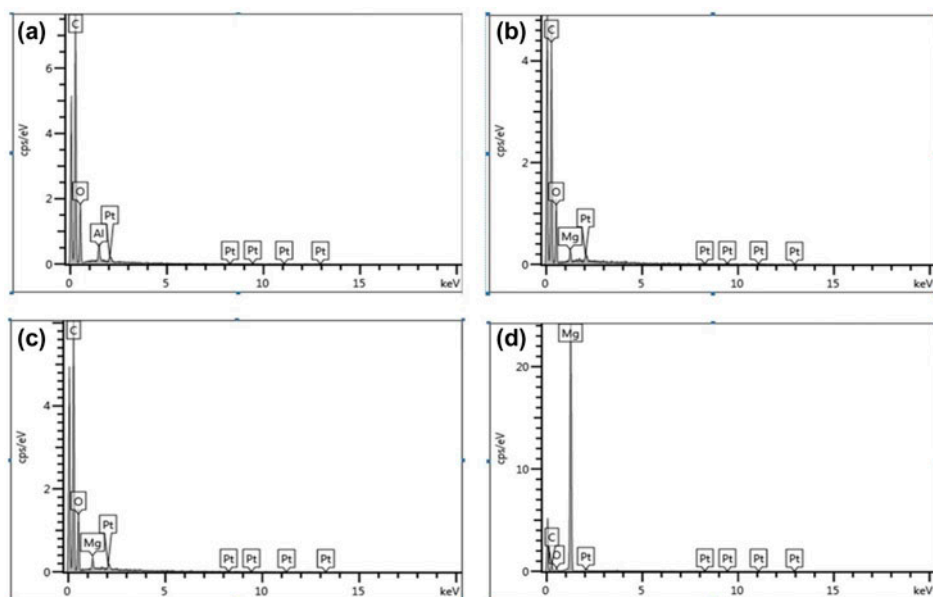


Figure 2. EDS elemental analysis pictures. (a) P(LLA-CL); (b) Mg/P(LLA-CL) (1:9); (c) Mg/P(LLA-CL) (1:4); and (d) Mg/P(LLA-CL) (1:2).

Table 2. Weight percentage and atomic percentages of Mg in the nanofiber scaffolds.

Sample	S1 (%)	S2 (%)	S3 (%)	S4 (%)
M% (Mg)	0	10.00	20.00	33.33
Wt.% (Mg)	0	12.08	19.18	36.54
At.% (Mg)	0	8.73	13.90	23.42

Notes: M% (Mg): mass content of Mg in prepared Mg/P(LLA-CL) electrospinning solutions.

Wt.% (Mg): weight percentage of Mg in Mg/P(LLA-CL) nanofiber scaffolds detected via EDS.

At.% (Mg): atomic percentage of Mg in Mg/P(LLA-CL) nanofiber scaffolds.

strength of the nanofiber scaffolds when the proportion of Mg increases beyond a certain value. In brief, the mechanical property of Mg/P(LLA-CL) nanofibers is acceptable to provide temporary support for tissues and organs.[37]

3.4. Degradation behavior

3.4.1. Weight loss of nanofiber scaffolds after degradation

The weight loss of nanofibers during degradation is shown in Figure 4. It can be seen that the weight loss of P(LLA-CL) nanofibers is the lowest at every time point and changes little during the 12 weeks. However, Mg/P(LLA-CL) nanofibers experience much higher weight loss than P(LLA-CL) nanofibers. Furthermore, the weight loss increases with the increasing of Mg content in Mg/P(LLA-CL) nanofibers. In degradation process, the weight loss was caused by the process that some soluble oligomeric compounds from the surface of polymer could be diffused and dissolved in the degradation medium, which were generated by the hydrolysis scission of polymer chains.[14] As the block copolymer of PLLA and PCL, P(LLA-CL) is composed of different chain

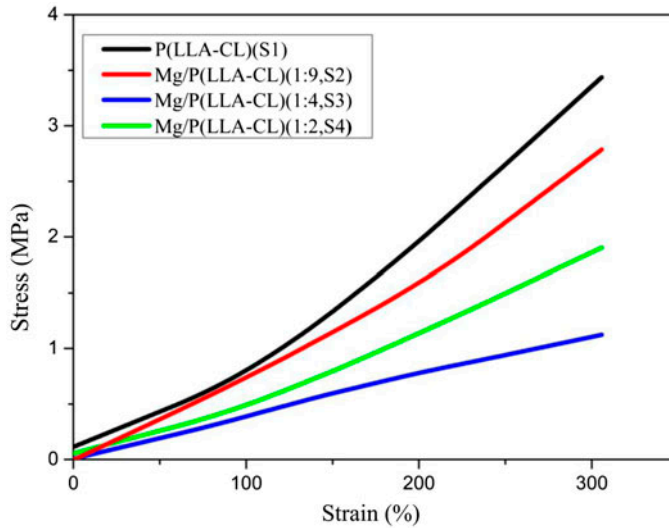


Figure 3. Mechanical property of the nanofiber scaffolds.

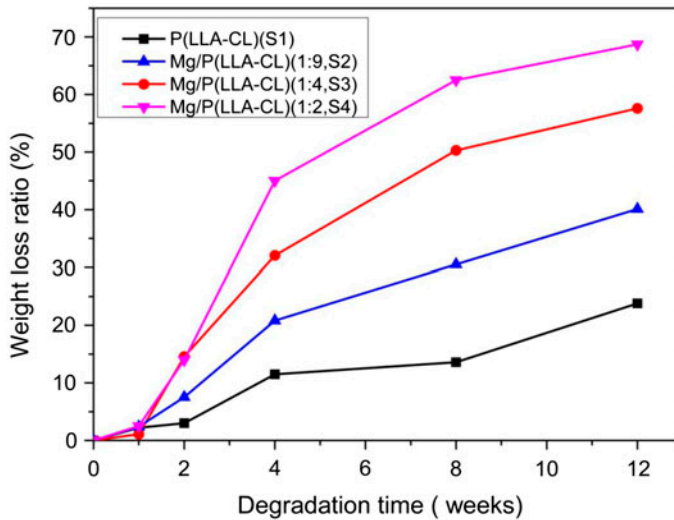
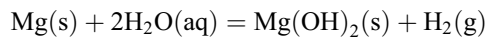
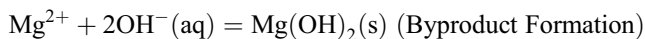
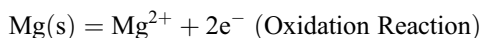


Figure 4. Weight loss of nanofiber scaffolds during degradation.

segments, these segments will give rise to the faster degradation rate than those of PLLA and PCL.[38] In addition, the degradation rate of Mg/P(LLA-CL) nanofibers were accelerated by the fast corrosion of Mg and magnesium ions released from Mg/P(LLA-CL) nanofibers.[35] Mg corroded in aqueous materials by several different oxidation–reduction reactions, which would yield magnesium hydroxide and hydrogen gas evolution.[37] The reactions are given as below [39,40]:



The Contributing Half-cell reactions consists of:



Due to the existence of Mg, water in the degradation medium (PBS solutions) would react (Reduction Reaction) and produced OH^{-} . The degree of these oxidation–reduction reactions is intense. Additionally, some Mg particles aggregated on the surface of nanofibers. Therefore, a lot of Mg particles could react in the degradation medium. As a result, this type of composite nanofibers degraded faster than pure nanofibers did and could be used as scaffold materials for short-term implantation.[37]

3.4.2. Morphology change of nanofiber scaffolds after degradation

The SEM images of nanofibers after degradation for 1, 2, 4, 8, and 12 weeks are shown in Figures 5–9. From the images, it is testified that the Mg micro-particles blended in

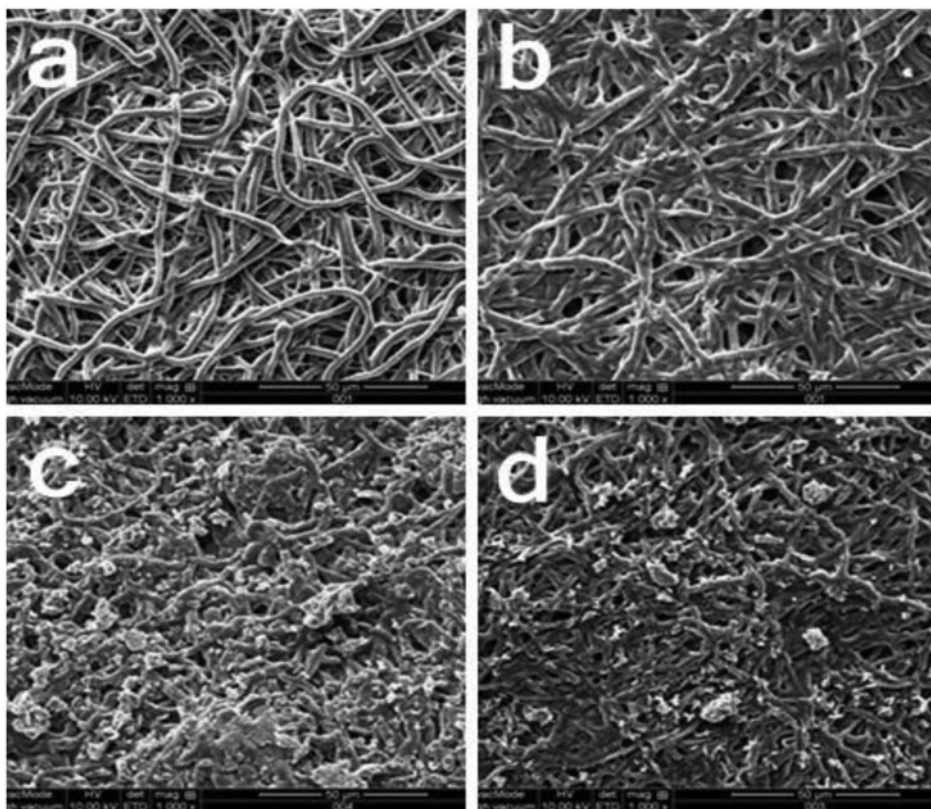


Figure 5. Morphologies of nanofibers scaffolds after degradation for 1 week. (a) P(LLA-CL); (b) Mg/P(LLA-CL) (1:9); (c) Mg/P(LLA-CL) (1:4); and (d) Mg/P(LLA-CL) (1:2).

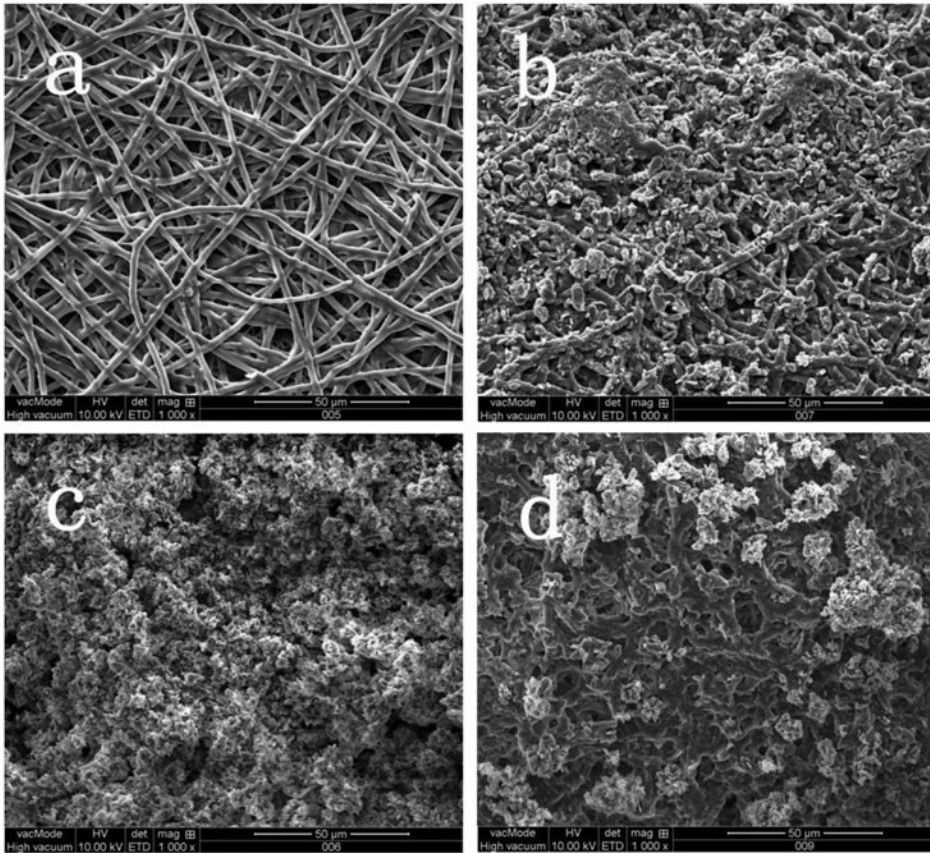


Figure 6. Morphologies of nanofibers scaffolds after degradation for 2 weeks. (a) P(LLA-CL); (b) Mg/P(LLA-CL) (1:9); (c) Mg/P(LLA-CL) (1:4); and (d) Mg/P(LLA-CL) (1:2).

the P(LLA-CL) affects the morphology of nanofibers. The surface structure of P(LLA-CL) nanofibers changed little after degradation for one week (Figure 5(a)). After degradation for two weeks, P(LLA-CL) nanofibers swelled lightly and parts of fibers adhered obviously (Figure 6(a)). After degradation for four weeks, the adhesion of nanofibers was severe (Figure 7(a)) and nanofibers were beginning to melt. Moreover, it was reported that polymer chains could be mobilized when the glass transition temperature (T_g) is close to or below the degradation temperature so that the fibers tend to ‘melt’ together to reduce the surface tension.[41,42] Therefore, the phenomenon of melt may be attributed to the good elasticity and the lower polymer T_g of the P(LLA-CL).[14] In contrast, the morphologies of Mg/P(LLA-CL) nanofibers degraded remarkably. After one week, the Mg/P(LLA-CL) nanofibers tended to be as molten as the pure P(LLA-CL), because Mg mainly dispersed P(LLA-CL) matrix with more content of P(LLA-CL) in Mg/P(LLA-CL)-blended nanofibers. Some fibers began to break up and the extent of adhesion had been severe (Figure 5(b)–(d)). When the degradation had lasted for two weeks, large areas of fracture appeared on Mg/P(LLA-CL) (1:4 and 1:2) nanofibers. The fiber membranes dissolved and the original fiber morphologies were destroyed (Figure 6(c) and (d)). It is obvious that Mg/P(LLA-CL) (1:2) with the

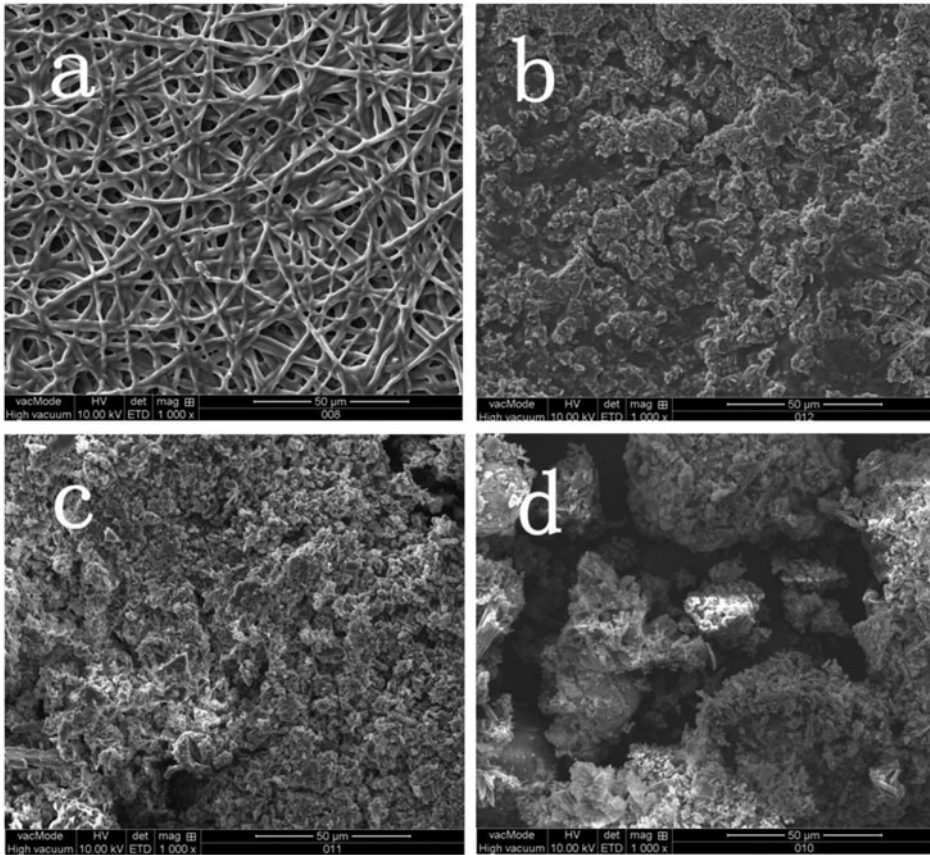


Figure 7. Morphologies of nanofibers scaffolds after degradation for 4 weeks. (a) P(LLA-CL); (b) Mg/P(LLA-CL) (1:9); (c) Mg/P(LLA-CL) (1:4); and (d) Mg/P(LLA-CL) (1:2).

highest proportion of Mg had the most intense degradation behaviors (Figure 6(d)). After having degraded for four weeks, Mg/P(LLA-CL) (1:9) lost the morphology features of fibers (Figure 7(b)). Several pores appeared on the surface of Mg/P(LLA-CL) (1:4) nanofibers (Figure 7(c)). Furthermore, Mg/P(LLA-CL) (1:2) had become powders thoroughly (Figure 7(d)). As the degradation proceeded to eight weeks, Mg/P(LLA-CL) (1:4 and 1:2) could not be observed by SEM anymore because the fibers had become tiny debris. It could be seen that P(LLA-CL) and Mg/P(LLA-CL) (1:9) degraded almost completely after 12 weeks (Figure 8(c) and (d)). This demonstrates that adding Mg particles accelerates the degradation rate of the nanofibers greatly.[35] Moreover, the higher the proportion of Mg particles in the nanofibers is, the faster the degradation rate of the fibers is. As demonstrated before, the main reasons are the several different oxidation–reduction reactions caused by Mg particles and the loose structure of composite nanofibers. That is why after Mg in the Mg/P(LLA-CL) scaffolds degraded in the solutions rapidly, the morphologies of Mg/P(LLA-CL) composite fibers would melt, dissolve, and break up.

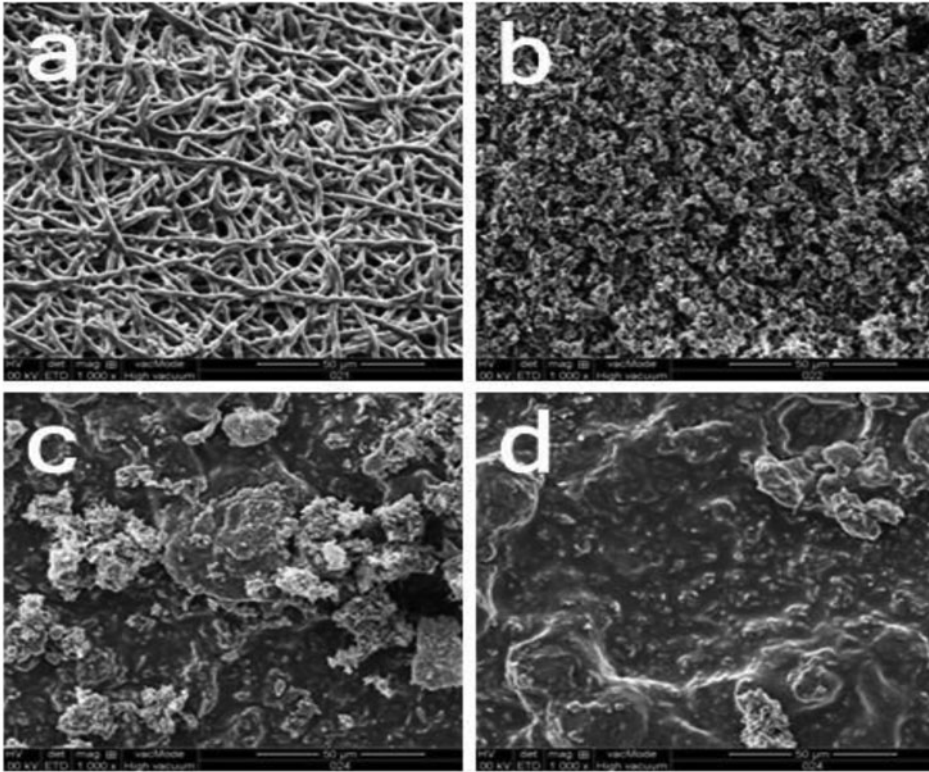


Figure 8. Morphologies of nanofibers scaffolds after degradation for 8 and 12 weeks. (a) P(LLA-CL) (8w); (b) Mg/P(LLA-CL) (1:9, 8w); (c) P(LLA-CL) (12w); and (d) Mg/P(LLA-CL) (1:9, 12w).

3.4.3. The pH value of the degradation solution

The pH value of the degradation solutions at different time points are shown in Figure 9. The pH value of P(LLA-CL) degradation medium decreased with the increase of degradation time, since soluble acid oligomers were diffused and dissolved in the degradation medium from the nanofiber scaffolds matrix. In particular, the carboxyl ($-\text{COOH}$) of oligomers contributes to the pH decline of the degradation medium.[14] But the pH value of Mg/P(LLA-CL) degradation medium increased rapidly at the beginning of the degradation, and the more Mg content led to higher pH value. The reason is that the Mg in Mg/P(LLA-CL) nanofibers made the solution alkaline through oxidation–reduction reactions.[16,43,44] But, after degradation for four weeks, pH value of Mg/P(LLA-CL) degradation medium began to decrease. As the degradation proceeded, the alkaline degradation medium was neutralized by the acidic groups produced by the degradation of P(LLA-CL). The hydrolysis of polymers would last until the P(LLA-CL) fibers degraded completely, however, the oxidation–reduction reactions of Mg were severer and would reach the point of saturation after a certain time.

3.5. Results of cytocompatibility test via MTT

MTT assay of cell proliferation on the nanofibers is shown in Figure 10. PIECs could proliferate on all substance, but they proliferated with different rates on different

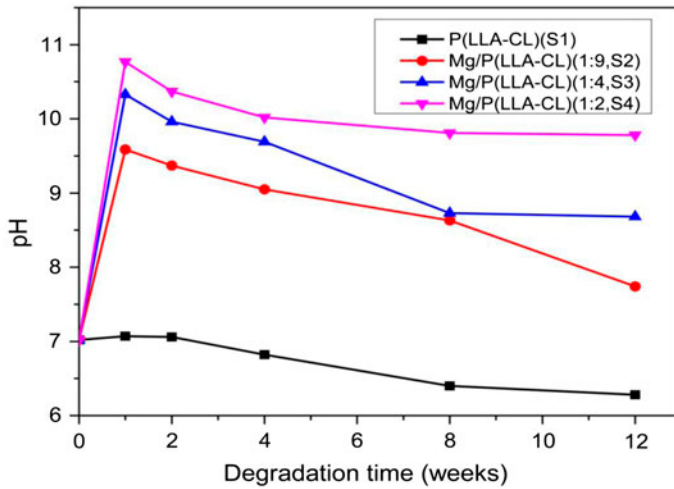


Figure 9. Change of pH value of nanofiber scaffolds after during the degradation.

substances. PIECs proliferated on P(LLA-CL) nanofibers with the highest rate. The MTT absorbance of P(LLA-CL) nanofibers is a little higher than the culture coverslips, demonstrating that P(LLA-CL) nanofiber scaffolds have good cytocompatibility. With Mg blended in P(LLA-CL) nanofibers, cell proliferation was inhibited. MTT absorbance is lower with the addition of Mg content in Mg/P(LLA-CL) nanofibers. The high pH may be the main factor, because the environment with strong base is not conducive to the growth of most cells. Fortunately, the high pH could not last long, so certain MTT absorbance of Mg/P(LLA-CL) fibers could be seen in the figure. Especially, the Mg/P(LLA-CL) (1:9) fibers possess the best cytocompatibility among the samples of composite nanofibers, which are suitable as temporary scaffolds.[37]

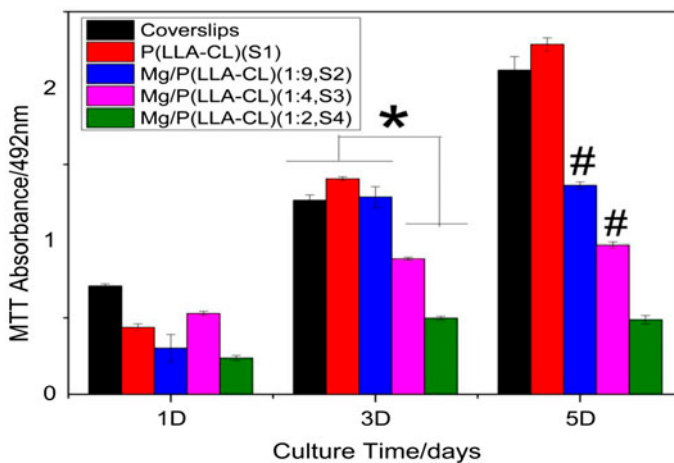


Figure 10. Results of cytocompatibility of nanofiber scaffolds at 1, 3, and 5 days via MTT. *Indicates a significant difference from others ($p < 0.05$); # indicates a significant difference from the 1st, 2nd, and 5th column datum ($p < 0.05$).

4. Conclusions

In this study, Mg and P(LLA-CL) could be electrospun to composite nanofiber scaffolds with different mass ratios successfully. Compared with pure P(LLA-CL) nanofibers, the average elongation at break of composite nanofiber scaffolds changed little, while the average tensile strength declined obviously. In addition, the pH value of Mg/P(LLA-CL) degradation medium was higher than that of P(LLA-CL) degradation medium and it increased with the increase of the proportion of Mg in the composite nanofibers. Additionally, after blending Mg, the degradation rate of P(LLA-CL) was accelerated. Furthermore, the Mg/P(LLA-CL) nanofibers had less amount of PIECs on the surface than pure P(LLA-CL) nanofibers had. Therefore, the Mg/P(LLA-CL) composite scaffolds may give neutral envelopment during their degradation, meanwhile, show acceptable mechanical property and cytocompatibility. Ongoing studies will focus on looking for more appropriate mass ratio of Mg and P(LLA-CL) for optimizing the properties of Mg/P(LLA-CL) composite scaffolds.

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