Incorporation of tripolyphosphate nanoparticles into fibrous poly-(lactide-co-glycolide) scaffolds for tissue engineering

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Poly(lactide-co-glycolide) (PLGA) has been widely used for scaffolding materials in tissue engineering. It degrades mainly via hydrolysis of the ester bonds into lactic acid and glycolic acid leading to the decrease in pH of the surrounding microenvironment. The current study was designed to quickly neutralize the acidic degradation products of PLGA fibrous scaffolds by incorporating tripolyphosphate (TPP) nanoparticles into PLGA fibers. A homogeneous mixture of PLGA and TPP was first obtained by water-in-oil emulsion–dispersion followed by freeze-drying. The dried blend was melt-spun to yield fibers which were processed into scaffolds and subsequently immersed into phosphate-buffered saline (PBS) to verify the degradation properties. The pH of the saline was monitored for a duration of 80 days. The amount of TPP was optimized to obtain a PLGA based scaffolds without acidic degradation problems. Cellular compatibility of the modified and pristine scaffolds was evaluated using rabbit adipose-derived stem cells (rASCs). It was shown that TPP particles within the fibers were roughly 100 nm in diameter and mainly located inside fibers instead of on the superficial layer. The acidic degradation of PT-16 and PT-64 (PT-X is termed when the monomer molar ratio of TPP to PLGA was 1:X) was significantly improved as the pH values of their respective solutions were maintained in a well neutralized state during the degradation. PT-64 and PT-16 scaffolds could well support the attachment and proliferation of rASCs. Hence, the incorporation of TPP nanoparticles via an emulsion–dispersion method could be an effective strategy to improve/adjust the acidic degradation of PLGA and further pave the way for clinical applications of such polyesters.

1. Introduction

Tissue engineering is an interdisciplinary and multidisciplinary field that aims at developing biological substitutes to restore, maintain, or improve tissue function. Scaffolding materials for tissue engineering can be any biomimetic biomaterials that mimic one or multiple characteristics of natural extracellular matrix (ECM)[1], supporting cell attachment, proliferation, differentiation, and neo-tissue generation [2].

To fulfill the diverse needs in tissue engineering, various materials have been exploited as scaffolds for tissue regeneration. Amongst the different classes of biodegradable polymers, the thermoplastic aliphatic polyesters like poly(lactide) (PLA), poly(glycolide) (PGA), especially their copolymer, poly(lactide-co-glycolide) (PLGA), have attracted immense interest due to their favorable properties such as good biocompatibility, suitable biodegradability, and mechanical strength [3]. They have been extensively investigated for applications in drug release, gene delivery [4–6] and engineering different types of tissue including cartilage, blood vessel and tendon [7–10].

In addition to their popularity in this biomedical field, their degradation behaviors have also been characterized thoroughly. It was shown that these polyesters degrade primarily in the body via chemical hydrolysis of the hydrolytically unstable ester bonds yielding oligomers with carboxyl end groups, or lactic acid as well as glycolic acid [11,12]. However, the main concern is the pH decrease in the milieu around the materials resulting from the accumulation of those acidic degradative products. The initially yielded acids are capable of catalyzing hydrolysis of other ester bonds, a phenomenon called autocatalysis [12]. If the acids cannot be freed away from the bulk material, or the accumulation of acids...
occurs within the scaffolds or devices based on these polyesters, the so-called bulk degradation would be induced. Hollow structures with extremely acidic inner environment are formed as a result [13].

The above phenomena will affect cell growth, differentiation, ECM formation and tissue regeneration. When implanted in bodies, non-specific inflammation of the host usually occurs in the early period [14,15]. It was also postulated that the inflammation reaction derived from PGA degradative products could be a major factor leading to a failure of generating human ear cartilage in a porcine model of subcutaneous implantation, although the same material had succeeded in engineering the same tissue in a nude mouse [16]. Hence, the clinical application of PGA, PLA, and PLGA is thus limited by their acidic degradation behaviors. Although both in vivo and in vitro degradations of PGA or PLGA polymers have been extensively investigated [11–13,17], it remains to be figured out how to eradicate the acidic influences from degradation and further enhance the biocompatibility of these materials, especially for devices and scaffolds designed for immunocompetent animals or human bodies.

It was reported that amine groups were used to modify the branched structure of PVA-g-PLGA polyester [18], typically realized by co-polymerization or functionalization of the polymeric side chains with positively charged groups via covalent attachment. It was effective as the neutralization occurred immediately during degradation and the pH could be maintained close to 7.4. However, the processing and mechanical properties of the resulting polymers were significantly changed resulting from such kind of modification [1]. Alternatively, surface modification can be used as an effective method to keep the surface pH to nearly neutral, without changing the bulk properties of materials. But it is only effective on the surface and the functional group will soon be washed off at the beginning of the degradation.

In this study, we developed a methodology to modify such types of biodegradable polymers. PLGA was chosen as a typical model to assess the effectiveness of such method. Sodium tripolyphosphate (TPP), a kind of salt with strong buffering capacity was chosen to modify PLGA. An emulsion method was applied to homogenize TPP in PLGA matrix. The resulting composite after being dry uniform bulk blend. The ratio of TPP to PLGA was calculated based on the monomer molar unit in PLGA could produce one carboxyl group (the main factor that leading to the decrease in the surrounding pH) and one effective unit of phosphate salt group can neutralize one unit of acid theoretically (TPP contains 5 effective units). The monomer molar ratio of TPP to PLGA was then varied as specified in Table 1 to yield different bulk blends for subsequent fiber formation and scaffold preparation.

### 2.3. Preparation and characterization of PLGA/TPP fibers

The above blends (PLGA/TPP) were crushed and melt-spun using a DACA MicroCompounder (USA) to obtain PLGA fibers incorporated with different amounts of TPP. The PLGA/TPP blends were first dried at 50 °C in a vacuum drying oven for 12 h before spinning. The temperature in the extruder of the MicroCompounder was set at 130 °C. The circular single spinneret orifice for extruding the fibers was 1 mm in diameter. PLGA/TPP blend of 5 g was fed into the chamber and the extruder was run for 10 min with the screw speed set at 100 rpm. A customized wind-up unit provided an automated traverse motion for collecting the melt-spun monofilament fibers at a winding speed of around 100 m/min. The composite fibers with different monomer molar ratios of TPP to PLGA as 1:1 were termed as PT-X as detailed in Table 1.

XPS analysis of the fiber surface was made on an Axis HSI spectrometer (Kratos Analytical Ltd.) using a monochromatized AlKα X-ray source (1486.6 eV photons) at a constant dwell time of 100 ms and a pass energy of 40 eV. The anode voltage was 15 kV and the anode current was 10 mA [21]. The pressure in the analysis chamber was maintained at 7 × 10⁻⁶ Pa or lower during each measurement. The fibers were mounted on standard sample stubs by means of double sided adhesive tape. The core-level signals were obtained at a photoelectron takeoff angle of 90° (with respect to the sample surface). To compensate for surface charging effect, all core-level spectra were referenced to the C 1s hydrocarbon peak at 284.6 eV.

Scanning electron microscopy (SEM) was carried out on a JED JXA-8100 scanning electron microscope (Japan) to observe the surface morphology of the fibers. The samples were cleaned with a nitrogen dusting gun and sputter-coated with a thin film of gold for imaging purposes. Transmission electron microscopy (TEM) analysis was carried out on a HITACH H-800 TEM to determine the size and distribution of TPP particles within PLGA fibers. The TEM was operated at an accelerating voltage of 200 kV and the depth for investigation was 100 nm. The fibrous samples, after being embedded, were cut into ultrathin cross-sections of 70 nm in thickness for TEM observation. Thermogravimetric analysis (TGA, TG 209F, Netzsch, Germany) of PLGA/TPP fibers was carried out to confirm the relative ratio of PLGA to TPP. The tensile strength of the fibers was evaluated using a universal tester (Instron 5542, USA) at a crosshead speed of 5 mm/min in the ambient condition with a temperature of 20 °C and a relative humidity of 50% (the normal condition usually maintained in our lab where the INSTRON machine is located). To minimize the effect of degradation in air, the fibers were freshly prepared and stored in a dry box under reduced pressure for less than 5 days before the mechanical test. Fibers with a gauge length of 20 mm were evaluated and the fiber extension was approximated from the crosshead movement. Data from the load deformation and stress–strain curves were recorded. The tensile stress at maximal load was obtained from these data for each sample. Four representative groups of samples including PLGA, PT-64, PT-16, and PT-6 were subjected to the mechanical test. The results were normalized to the one of pristine PLGA fibers. All values are the mean of four independent experiments.

### 2.4. Scaffold preparation and in vitro degradation test

As described previously [10], 100 mg of fibers were pressed into a mesh of approximately 10 mm in diameter and 2 mm in height. The resulting scaffolds were first sterilized using 75% ethanol for 20 min and rinsed with sterile phosphate-buffered saline (PBS) solution (pH 7.4, 0.1 M) 3 times. The excess liquid on scaffold surface was removed by filter paper. Then the scaffolds were immersed in 10 ml sterile PBS at 37 °C. Disposable sterile polypropylene centrifuge tubes (15 ml) were used as test vessels. The PBS solution was not changed throughout the duration of in vitro degradation. The pH of the solution with or without scaffolds was measured daily using a pH meter (DELTA 320, METTLER TOLEDO, Switzerland). Four representative groups of samples including PLGA, PT-64, PT-16, and PT-6 were subjected to the in vitro degradation test. Each group included 3 samples.

### Table 1

<table>
<thead>
<tr>
<th>Samples</th>
<th>Monomer molar ratio of TPP to PLGA</th>
<th>TPP/PLGA (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. PLGA</td>
<td>0:1</td>
<td>0:1</td>
</tr>
<tr>
<td>B. PT-64</td>
<td>1:64</td>
<td>17.7:1</td>
</tr>
<tr>
<td>C. PT-32</td>
<td>1:32</td>
<td>35.5:1</td>
</tr>
<tr>
<td>D. PT-16</td>
<td>1:16</td>
<td>70.9:1</td>
</tr>
<tr>
<td>E. PT-10</td>
<td>1:10</td>
<td>113.5:1</td>
</tr>
<tr>
<td>F. PT-8</td>
<td>1:8</td>
<td>141.9:1</td>
</tr>
<tr>
<td>G. PT-6</td>
<td>1:6</td>
<td>189.2:1</td>
</tr>
</tbody>
</table>
2.5. Isolation and culture of rabbit adipose-derived stem cells

The experimental protocol was approved by the Animal Care and Experiment Committee of Shanghai Jiaotong University School of Medicine. Rabbit adipose-derived stem cells (rASCs) were isolated and cultured as previously described [22,23]. The extracted cells were plated on 100 mm culture plates (Falcon, USA) with a density of \( 1 \times 10^5 \) cells/cm\(^2\) and incubated at 37°C in a humidified atmosphere containing 95% air and 5% carbon dioxide with the Dulbecco’s modification of Eagle’s medium (DMEM medium) containing 10% fetal bovine serum (FBS, HyClone, USA), L-glutamine (292 mg/L), penicillin (100 U/ml), streptomycin (100 mg/L), and ascorbic acid (50 mg/L) changed twice a week. When 90% confluence was reached, the primary cells were detached using trypsin–EDTA (0.025% and 0.02%, respectively) followed by subculture at 1:6 (e.g. 1:4), the continuity of the fibers was not good. The poorly formed fibers were easy to be broken. Hence, the highest monomer molar ratio of TPP to PLGA used was 1:6 in this study.

XPS analysis of some typical fibers (PLGA, PT-16, and PT-6) was carried out to observe the elements present on the superficial layer of these fibers. The XPS P 2p core-level spectra of the fibers with or without the incorporation of TPP were shown in Fig. 2. Since there is no phosphor in pristine PLGA, no phosphor component can be detected as shown in Fig. 2B. Trace amount of phosphor can be detected in the fibers of PT-16 and PT-6 (Fig. 2D and F). From the wide scan, only C 1s and O 1s core-level spectra can be observed in all of the fibers (Fig. 2A, C and E), while the intensity of O 1s signal increased relative to the one of C 1s signal after the incorporation of TPP. The above phenomena indicated that the superficial layer of the fibers was not enriched with TPP, although its presence can be ascertained (usually the probing depth of the XPS technique is ~10 nm for an organic matrix [26]). This ensures that during the degradation process, the TPP inside the PLGA fibers would not be soon washed off with the proceeding of hydrolysis of the surface polymeric molecules.

The microstructure of the above fibers was examined using SEM (Fig. 3). For PLGA, PT-64 and PT-16, most of the fiber surface was

Cells from three individual rabbits were subjected to the above in vitro tests. Images of the above SEM or CLSM were representative of three independent experiments. The in vitro proliferation data were average values from three individuals, and each of them was the mean of triplicate experiments.

2.8. Statistical analysis

The data is expressed as means ± standard deviation. Student t-tests were used to determine statistical significance between groups, and \( p < 0.05 \) was considered significant.

3. Results and discussion

3.1. Characterization of PLGA fibers

PLGA fibers with different amounts of TPP were prepared by melt-spinning. The resulting fibers are shown in Fig. 1 with the monomer molar ratio of TPP to PLGA varied and specified in Table 1. Grossly, the fibers were all pliable and smooth with similar whitish appearance (Fig. 1). The diameter of all fibers was around 45 μm and the length of each fiber could reach a few meters or even longer depending on the amount of feed materials unless broken accidentally. With the increase in the amount of TPP, it became difficult to form fibers. When the monomer molar ratio of TPP to PLGA exceeds 1:6 (e.g. 1:4), the continuity of the fibers was not good. The poorly formed fibers were easy to be broken. Hence, the highest monomer molar ratio of TPP to PLGA used was 1:6 in this study.

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The microstructure of the above fibers was examined using SEM (Fig. 3). For PLGA, PT-64 and PT-16, most of the fiber surface was
quite smooth, while an accidented topography could also be located randomly and infrequently as shown in Fig. 3A–C. However, as to PT-6 (Fig. 3D), the whole fiber surface became quite rough probably due to the high concentration of TPP incorporated. It seems that the surface roughness of the topography appears to increase with increasing TPP level.

The size and inner distribution of TPP particles in the PT-16 and PT-6 fibers were visualized by TEM analysis as shown in Fig. 4. It can be seen that the size of most TPP particles inside the PT-16 fibers was less than 100 nm (inset of Fig. 4A), and the distribution is quite uniform (Fig. 4A). With the increase in the amount of TPP particles, some particles became aggregated as shown in the inset of Fig. 4B, and the overall dispersion remained uniformly across the fiber (Fig. 4B). Moreover, the size was still at around 100 nm. Together with the XPS results, it was further confirmed that TPP particles mainly located inside the fibers instead of on the fibrous surface.

TGA studies were carried out for the modified (PT-64, PT-16 and PT-6) PLGA fibers. Pristine PLGA fibers and TPP were also subjected to the same test to serve as controls. The fibers with different amounts of TPP incorporated gave their distinctive TGA curves (Fig. 5), which could provide indications of the relative amounts of PLGA and TPP in the fibers. The TGA curve of the pristine PLGA fibers showed a weight loss of about 99% after the fibers being heated to 600 °C, and the weight loss was initiated at ~300 °C and ended at ~400 °C. The curve of TPP showed a weight loss of 30% at around 100 °C. The TGA curves of PT-64, PT-16 and PT-6 showed a two-stage combinational weight loss upon heating in air. The first stage showed a weight loss at around 100 °C, characteristic weight

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**Fig. 2.** XPS wide scan, P 2p core-level spectra of the pristine PLGA, PT-16, and PT-6 fibers prepared by melt-spinning process.

**Fig. 3.** Scanning electron micrographs of (A) PLGA, (B) PT-64, (C) PT-16, and (D) PT-6 fibers. Scale bars: 10 μm.
loss of TPP. The second stage of weight loss stepped from 300 °C to 400 °C, similar to that of pristine PLGA. The residual weight of TPP and PLGA was ~70% and ~1%, respectively. A trend of increase in the residual weight was observed from the TGA curves in the order of PT-64, PT-16 and PT-6 (2%, 6%, and 16% respectively). The above results were in well consistence with the ingredients of different fibers as specified in Table 1.

3.2. Tensile strength of PLGA fibers

It is important that the modified fibers could still retain their original mechanical property. Thus, the tensile strength of a single fiber was measured to estimate the mechanical property of the composite fibers. The value was also compared with that of pristine PLGA and expressed as the relative tensile strength. The effect of the presence of TPP particulates in fibers was then shown in Fig. 6. PT-64 possessed around 91.42 ± 4.41% of that of pristine PLGA, while PT-16 reached close to 68.11 ± 2.30%. With the further increase in the amount of TPP, the tensile strength also decreased. The tensile strength of PT-6 was decreased to only around 43.71 ± 4.86%. The relative tensile strength of PT-64 was significantly higher than that of PT-16 (p < 0.05), also significant difference was observed between PT-16 and PT-6 (p < 0.05). Obviously, the degree of such compromising effect depended largely on the amount of TPP. Moreover, as the hydrophilic properties of the PLGA/TPP composite fibers are a function of TPP content, it is possible that the differences in tensile strength may partially come from the different water contents.

3.3. In vitro degradation of PLGA scaffolds

The above four fibers were processed into scaffolds which were in a size of about 10 mm in diameter and 2 mm in height as shown in Fig. 7A and B. The degradation behaviors of different PLGA discs were then shown in Fig. 7B and C to assess the effect of TPP contents on in vitro degradation of PLGA. Pre-wetting of PLGA based scaffolds to ensure all surfaces of the materials within the scaffolds were in contact with the PBS solution was very important as PLGA is relatively hydrophobic as proposed by Wu and Ding [17]. Hence, the scaffolds before degradation were immersed in 75% ethanol for 20 min not only for sterilization but also for wetting purposes as did by Lu et al. [27]. Although ethanol can render a plasticizing effect on PLGA, no visual and microscopic deformation of the fibers after the current ethanol treatment (75%, 20 min) by SEM could be observed (data not shown).

During degradation, the morphology of each type of scaffolds changed via a different temporal profile. As shown in Fig. 7B, after degradation for 1 week, all scaffolds except PT-6 shrank obviously. At this time point, the scaffold of PT-6 began to swell after initial shrinkage which occurred early than the other samples. When observed at 7 weeks after degradation, the samples of PLGA, PT-64 and PT-16 expanded extensively. The PT-6 at this time point became a little bit whiter. When it came to 10 weeks, PLGA and PT-6 almost vanished and only some fibrous debris can be observed at the bottom of the testing vials. On the other hand, the structure and morphology of PT-64 and PT-16 still remained. PT-64 exhibited
A little bit whiter and transparent appearance than PT-16 at this time point. A few days later, PT-64 also vanished similarly as that of PLGA or PT-6 (data not shown), while PT-16 vanished after PT-64. Consistent with the current study, a dimensional decrease of PLGA porous scaffolds was usually observed at the very early stage of degradation [17]. Such a decrease would be followed by an expansion in dimension, and after that, the scaffolds gradually became brittle and a little bit whiter as observed by Wu et al. [17]. Obviously, the time sequence of morphological change of the four scaffolds occurred in the order of PT-6, PLGA, PT-64 and PT-16.
For the pristine PLGA, the pH value of the solution decreased gradually after being immersed into the solution. At the end of 5 weeks, the pH had decreased to around 6.00. Since then, the decrease rate in pH was slowed down till 57 days after degradation. After that stage, the decrease rate rapidly increased with a sudden decrease in pH being observed in some dozens of days. The pH value was less than 3.00 at 70 days. With the further increase in the degradation time, the hydrolysis rate was slowed down again and the pH had decreased to 2.70 at the end of the testing period (80 days). As to the sample of PT-6, the pH had decreased at a relatively constant rate. After degradation for 80 days, the pH had decreased to 2.50. At each time point, the pH of the solution containing PT-6 was far lower than that of pristine PLGA. Only minor decrease in the pH value of the solution containing PT-16 was observed during the whole degradation period. After 7 weeks, the pH of PT-16 group was still maintained higher than 7.00. At the end of 80 days, the pH was 6.07. During the whole degradation period, no sudden decrease in the pH value was observed. As to the PT-64 group, after a gradual decrease in the pH value of the solution for 10 weeks, the pH had decreased to 6.32 which was significantly higher than that of PLGA at the same time point (pH = 3 for pristine PLGA, p < 0.05). After that period, the degradation rate was increased dramatically and the pH value soon decreased to 3.40 in 10 days.

For the pristine PLGA, a three-stage process was previously proposed for in vitro degradation according to the characteristic changes of some properties [17]. Stage I was characterized by a decrease in the dimensions of the porous scaffolds, and usually this stage lasted for about 4 weeks but depending on the different initial formulations. In stage II, a dramatic decrease in mechanical properties was happened due to the vast production of low molecular weight degradation products [17]. These oligomers most probably still remained within the matrix and initiated the so-called autocatalysis without being seriously released into the surrounding medium. Therefore, pH decrease was not obvious in this period from ~5 weeks to 8 weeks as observed in this work. Stage III was characterized by dramatic weight loss and eventual disruption of the scaffold resulting from the diffusion and disolution of acidic degradation products into the medium [17]. Our data in Fig. 7 further demonstrated the above proposed in vitro degradation process of PLGA. The presence of TPP particles within the fibers of PT-64, PT-16 and PT-6 scaffolds would complicate the hydrolytic degradation of the polymers. First, it facilitated the water permeation and penetration into the scaffolds due to the osmotic force. Second, the release of these particles would increase the porosity and thereby increase the specific surface area of the scaffold. The above two factors would enhance the hydrolysis of these ester bonds of PLGA. On the contrary, the presence and release of TPP particles could neutralize the nearby acids produced from the hydrolysis, thus preventing the autocatalyzed hydrolytic reaction. The final measured pH value was thus a complicated interplay between the diffusion rates (of water, buffering particles, and reaction products) and the reaction rate (of polymer with water, autocatalyzed by the acidic reaction products). Furthermore, the release and diffusion of the TPP particles would partially depend on the size, distribution and uniformity of particles within PLGA matrix.

According to the results shown in Fig. 7, PT-6 obviously had an accelerated degradation and the pH decrease of the surrounding medium was worsened, probably due to the dominant effect of the first two negative aspects of TPP. From the pH decrease in the medium, a three-stage degradation process could not be distinguished any more for PT-6. However, it was interesting to notice that the acidic degradation of PT-64 and PT-16 was improved extensively, especially the sample of PT-16. In such case, the size and distribution of TPP in PT-64 and PT-16 may be suitable for achieving an optimized releasing and functioning balance. At the late stage of degradation, PT-64 also experienced a sudden but delayed pH decrease similar to that of pristine PLGA. This might result from the insufficient amount of TPP incorporated. In conclusion, it is now desirable to make PLGA devices or scaffolds with tunable or tailored degradation behaviors without serious unfavorable acidic influence using the current proposed TPP particles and a simple emulsion-mixing method. Such a design could also be applicable to PLGA scaffolds of other forms in addition to fibers as well as to other polyesters with a similar degradation mechanism.

In addition, other properties like weight loss, mechanical properties, molecular weight, and microscopic morphology were not addressed in detail during degradation in this study as our main focus was the issue of acidic degradation. Further study is ongoing in our lab to present such relative information. Moreover, since these materials are mainly used for scaffolding purposes in tissue engineering, cellular compatibility assays of these scaffolds were carried out as shown in the following section.

3.4. Attachment and distribution of rASCs on the scaffolds

According to the above degradation results, scaffolds of PT-64 and PT-16 were chosen for cell seeding. The results were also compared with that of pristine PLGA. SEM which allows for the visualization of cell morphology and ECM deposition on different fibrous scaffolds was carried out on samples after being seeded with rASCs for 1, 3, and 7 days. As shown in Fig. 8A1, B1 and C1, after 1 day, the cells exhibited a spindle-shaped morphology with extensive cytoplasmic processes that anchored themselves tightly to the surface of fibers. Cell–cell communication was already established (Fig. 8A1, B1 and C1). After 3 days of culture, abundant ECM deposition was found evidently surrounding all those fibers with layered cell clusters uniformly distributed along these scaffolds (Fig. 8A2, B2 and C2). When the culture time was extended to 7 days, vivid production of ECM was continuously enhanced over time for all samples (Fig. 8A3, B3 and C3). Cell layers were formed among the fibers and the scaffolds were covered almost completely with cellular layers. The above results indicated that PLGA scaffolds in such a fibrous form possessed good biocompatibility to support the initial attachment and subsequent proliferation of rASCs in vitro. Moreover, no obvious difference between the PLGA scaffolds with or without TPP could be observed.

The distribution and growth of rASCs along the fibers can be further visualized by confocal images of DIO pre-labeled rASCs at 1, 3 and 7 days after being seeded on the scaffolds (Fig. 9). For all the groups, at 1 day post-seeding, labeled cells which distributed in a dispersed way already spread homogeneously along the fibers and cell assemblies in the fibrous form were then observed at 3 days. The cell density increased vividly with time and a dense coverage over the fibers at 7 days post-seeding was obtained. Again, no visual difference in the cell distribution could be observed between PLGA and PT-16, or between PLGA and PT-64 groups.

3.5. Proliferation of rASCs on the scaffolds

To quantitatively evaluate the proliferation of rASCs seeded on these scaffolds and also distinguish the minor difference in the growth of seeded cells on PLGA scaffolds with or without TPP addition, DNA assay was carried using Hoechst 33258 dye at days 1, 3, 7 post-seeding (Fig. 10). It can be seen that the cell numbers kept on increasing with time on each scaffold. For the pristine PLGA, a 1.6-fold increase in the cell number was achieved after 7 days of culture, while 2.1-fold and 1.3-fold increases in the cell numbers
Fig. 8. SEM images of rabbit adipose-derived stem cells (rASCs) cultured on PLGA (A), PT-64 (B) and PT-16 (C) for 1, 3, and 7 days. Insets are the representatives of a lower magnification of SEM images. After been seeded for 1 day, the SEM results showed that cells attached well on the surfaces of each fiber with many filopodia (A1, B1, C1). After 3 days of culture, abundant ECM deposition was found evidently surrounding all those fibers (A2, B2, C2). Seven days after cell seeding, vivid production of ECM was continuously enhanced over time for all samples (A3, B3, C3). Bar scales: 20 μm for A, B, and C; 50 μm for insets.

Fig. 9. Confocal images of Dio-labeled rASCs on PLGA (A), PT-64 (B) and PT-16 (C) after been seeded for 1, 3 and 7 days. Confocal images revealed the distribution of cells along the fibers with vivid proliferation over time. Bar scales: 300 μm.
were achieved after 7 days of culture for PT-64 and PT-16, respectively. The cell number on PT-64 at 7 days was significantly higher than the respective one on PLGA ($p < 0.05$), whereas the one on PT-16 at 7 days was around 80% of that on PLGA ($p < 0.05$). These results indicate that TPP at a certain concentration in the PLGA fibers (PT-64) may enhance cell proliferation over the pristine PLGA. When the concentration of TPP was increased to 1/16, it may render a negative effect on cells. However, despite of the minor difference in the cell number, cells could still maintain at an active state as evidenced both from SEM and confocal images. It would be more valuable to confirm if a long-term follow-up could be carried out together with an in vivo study regarding to the neo-tissue formation of such modified scaffolds as the acidic degradation was not so obvious during the first week.

Adult mesenchymal stem cells (MSCs) isolated from the adipose, ASCs, were chosen for the above evaluation, as they hold strong promise for use as seed cells in tissue engineering and regenerative medicine due to their virtually multiple-lineage differentiation potential [28–30]. Moreover, their great therapeutic potential has been extensively acknowledged with remarkable successes in repairing defects of bone or cartilage, as well as other tissues in animal studies using tissue engineering techniques [31–33]. The attachment and proliferation assays of rASCs were then carried out to give preliminary evaluation of the cellular compatibility of PT-16, PT-64 and PLGA in order to address whether TPP could be used safely in a biological system involved in tissue engineering. The long-term scaffolding capacity would be addressed in our further study involving the specific differentiation of stem cells and neo-tissue formation as well as the degradation in vivo.

The reason why the issue of acidic degradation of PLGA scaffolds has not been addressed satisfactorily so far is due to the fact that agents applied to modify these scaffolds should not only be biocompatible, but also have to undergo the harsh preparation procedures. In our case, TPP could satisfy the above two criteria. However, the dissolution of TPP is extremely faster than PLGA hydrolysis when exposed to an open system simultaneously. This is also one of the main difficulties encountered when performing this work. Only if dissolution and release of most TPP could occur later than PLGA hydrolysis, would the neutralizing effect be effective. We thus applied the water-in-oil emulsion method under a high homogenizing speed of 30,000 rpm to disperse TPP within the bulk PLGA phase. This method guaranteed a homogeneous distribution of TPP at the size of 100 nm within the continuous PLGA phase. Moreover, this method ensured that only a small portion of TPP particles would be present on the surface of PLGA fibers during the spinning process, but depending on the amount of TPP used. Briefly, the size and distribution of TPP particles as well as the quantity would affect the relative rate of TPP dissolution, release and PLGA hydrolysis. Finally, the concentration of TPP was optimized and the optimized samples, PT-64 and PT-16, could support the attachment and proliferation of rASCs, especially the PT-64.

4. Conclusion

The major finding of this study is that TPP particles could be successfully incorporated into the PLGA fibers by means of W/O emulsion followed by melt-spinning process. The incorporated particles were in the nanoscale range of around 100 nm with a uniform distribution within the fibers instead of on fiber surface. These nanoparticles could drastically improve the acidic hydrolysis of PLGA fibers by neutralizing the acids produced from PLGA degradation during release. The final effect was largely depended on the amount of TPP used. The pH of the solution containing PT-16 scaffold could be maintained at a neutral state during the whole degradation period, while the presence of TPP in PT-64 scaffolds not only lessens the serious pH decrease of the surrounding medium, but also enhances the proliferation of cells seeded on it. Hence, the efficacy of the current design by choosing TPP as the functional molecules and W/O emulsion as the incorporating method was demonstrated. The clinical application of PLGA in tissue engineering and biological systems could be further promoted by modification with TPP to prevent acid-accumulation and eradicate subsequent bulk degradation.

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Appendix

Figures with essential color discrimination. Figs. 7 and 9 of this article have parts that are difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.03.004.

References


