PCL-PGLA composite tubular scaffold preparation and biocompatibility investigation

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ABSTRACT: The objective of this paper was to fabricate a biodegradable tubular scaffold for small diameter (d < 6 mm) blood vessel tissue engineering. The tube scaffold needed a porous wall for cell attachment, proliferation and tissue regeneration with its degradation. A novel method given in this paper was to coat a porous layer of poly (ε-caprolactone) (PCL) on the outside of a poly (glycolic-co-lactic acid) (PGLA with GA: LA = 90:10) fiber braided tube to give a PCL-PGLA composite. The PGLA tube was fabricated using a braiding machine by inserting a Teflon tube with the desired diameter in center of the 20 spindles, which are the carriers of PGLA fibers. Changing the diameter of the Teflon tube can vary the inner diameter of a braided PGLA tube. Thermally induced phase separation method was used for PCL solution coating on the surface of the PGLA braided tube. Controlling the polymer concentration, non-solvent addition and quenching temperature generated the pore structures, with pore sizes ranging from 10-30 µm. The fibroblast cells were seeded on the tubular scaffold and cultured in vitro for the biocompatibility investigation. Histology results showed that the fibroblast cells proliferated on the interconnected pore of the PCL porous layer in 1 week. (Int J Artif Organs 2006; 29: 790-9)

KEY WORDS: Blood vessel tissue engineering, PCL, PGLA fiber, Scaffold, Thermally induced phase separation

INTRODUCTION

Atherosclerotic vascular disease, such as coronary artery disease, and peripheral vascular diseases are caused by the progressive narrowing and hardening of the arteries over time. Currently, this remains a significant cause of morbidity and mortality worldwide (1). Approximately 500,000 coronary artery bypass surgeries are performed each year in the US, and this further emphasizes the seriousness of this disease. This surgical procedure, together with heart surgery, is second only to skin tissue transplants and outnumbers surgeries performed on the bone, cartilage, liver, pancreas and kidney. Coronary artery bypass surgery is the main procedure for affected blood vessels < 6 mm in diameter (2).

Replacement procedures for affected blood vessels include grafting with autologous veins and arteries such as the saphenous vein or the internal mammary artery. Both procedures are flexible, viable, non-thrombogenic and compatible. However, the mammary artery is not always the appropriate size or length, and surgery of the saphenous vein can result in degenerative varicose alterations that can lead to aneurysm formation when transplanted in a high-pressure arterial site. Secondly, autologous grafting involves harvesting the veins and arteries from sites that leave wounds, which can break down or become infected resulting in further compli-
cations. In addition, many patients do not have suitable veins for grafting due to pre-existing vascular disease or because the veins were harvested in prior vascular procedures. In such cases, patients are restricted to modest treatment modalities, which might result in a heart attack (myocardial infarction). Finally, significant morbidity and surgical costs are also important considerations associated with the harvesting of autologous vessels (1, 3, 4).

Venous and arterial allografts have also been experimented with as natural substitutes for affected blood vessels, but have generally been abandoned for clinical reasons because they show a high incidence of rejection, deterioration and complications.

For these reasons, various research groups have begun to explore the use of synthetic materials to fabricate a substitute blood vessel. Vascular prosthetic devices have been made from a wide variety of materials and includes Dacron, polytetrafluoroethylene or (PTFE) and polyurethane. Both Dacron and PTFE have been satisfactory in applications requiring large diameter (> 6 mm) vascular substitutes, while in applications of smaller diameters such as coronary bypass, thrombosis rates > 40% after 6 months of implantation, as well as blood coagulation on the luminal surface resulting in occlusion (closure of lumen), have been reported. Therefore, in an attempt to overcome these limitations, the engineering of cardiovascular tissue is being actively explored as a new multidisciplinary approach, harnessing the principles of engineering and life sciences to develop and to create completely autologous, living replacement structures like heart valves and blood vessels.

In tissue engineering, biodegradable polymeric scaffolds have been developed to serve both as supports and as templates for cell seeding to occur. Their biodegradability ensures that as cells differentiate to form tissues, they degrade leaving no traces in the body. Since the polymeric scaffold acts as a guide for cells to form tissues, it has to possess certain properties such as the stable tube shape for cell attachment, the biodegradability and porosity for cell proliferation. It was the purpose of this study to investigate some of the techniques that can be used to fabricate a biodegradable scaffold for engineering tissue in blood vessels. Based on reports, several methods have been used for the fabrication of tubular scaffolds. Wake et al fabricated a pliable, porous, biodegradable scaffold with poly (DL-lactic-co-glycolic acid) (PLGA) and poly(ethylene glycol) (PEG) blends, using a solvent-casting and particle-leaching technique (5). Niklason and Langer fabricated a vascular graft for in vitro engineering of blood vessels (6). In their study, non-cross-linked PGA mesh scaffolds were sewn into tubular forms with 6-0 uncoated Dacron sutures, which were then attached to an inner lining consisting of a thin PGLA film. Stitzel and Joel reported a new method for fabricating the scaffolds, which could supply bio-mimic environments for seeded cell growth and communication (7). In their study, collagen fiber was wound on a 4 mm metal mandrel, and a PLA nanofiber was deposited on the wound collagen fiber. Smooth muscle cells (SMC) were seeded on the scaffold of the nanofiber tube, which was supported by collagen fiber, and then cultured in vitro.

Seliktar et al conducted the experiments on in vitro blood vessel regeneration from collagen hydrogel (4). A collagen hydrogel tube suspended with SMC cells was fixed in a bioreactor with in vitro culture SMC collagen gel suspension at cycling extension strength. Circumferential orientation of SMC was found in the histology result, but during culture the collagen hydrogel shrank.

Watanabe et al in Japan prepared a tube scaffold by using polyglycolic acid (PGA) non-woven fabric sheet coated with poly (L-lactide-co-caprolactone) (8). It has been successfully used for aorta replacement in dogs. Shum-Tim et al also produced a PHA-PGA biodegradable tube with inner diameter of 7 mm for tissue engineering of aorta (9). These two methods are suitable for building biodegradable tubes with a diameter > 6 mm and with greater wall thicknesses.

In this study, we developed a new kind of biodegradable tube scaffold. It is a composite tube of braided PGLA coated with a porous poly (ε-caprolactone) (PCL). The braided PGLA can yield the tube shape with the inner diameter the same as the Teflon tube inserted in the center of the braiding machine. The porous PCL layer coated on the PGLA braided tube generated the ideal tube scaffold with a stable shape and a porous wall for cell seeding. This PCL-PGLA composite biodegradable tube has the potential for blood vessel tissue engineering both in vitro and ex vivo.

The specific objectives of this study were to develop synthetic biodegradable scaffolds for tissue engineering of blood vessels; to investigate and assess the techniques used to fabricate biodegradable scaffolds; to characterize the scaffolds in terms of their pore distribution, pore morphology, porosity and pore size; to conduct preliminary cell seeding on a scaffold to investigate its toxicity to cells, as well as on cell attachment and proliferation.
MATERIALS AND METHODS

Materials

Poly (glycolic-co-lactic acid) (PGLA, GA: LA = 90/10) fibers with single fiber diameter of 20 µm and 20 fibers in one yarn was purchased from Shanghai Tianchuen Biomaterials Ltd, P.R. China. PCL granules with an average molecular weight of 80,000 were purchased from Aldrich Chemical Company, US. 1,4 dioxane was from Reagent Chemical Services Ltd, UK. 3T3 mouse skin fibroblasts, Dulbecco Modified Eagle’s Medium (DMEM), fetal bovine serum, tissue freezing medium, penicillin and streptomycin were from Clonetics Inc, US.

PGLA tube braiding

Tubular braided PGLA constructs were formed by microbraiding on a circular braiding machine (model 102-C13, KOKUBUN, Japan) with 20 spindles (carriers). Six PGLA fiber yarns were wound on each of the 20 spindles of the microbraiding machine. The ends of the fibers from the spindles were then pulled to the center of the microbraiding machine. Braided tubes 4 mm in diameter were fabricated by inserting a Teflon tube with a diameter of 4 mm in the center of the microbraiding system. Changing the gear ratios can change the braiding angle of the tube scaffold.

Cloudy point of the PCL solution

PCL dioxane solution (5 mL) and PCL dioxane/water (90/10 v/v) solution were prepared each with concentrations ranging from 1-25% (w/v). The solution was heated to above 30°C to get a clear solution in a water bath, and then the temperature was reduced by 1°C each time until the new temperature was maintained for 1 hour. When the temperature was reduced down to 12°C, the sample was placed in the refrigerator with a thermometer. The same processes as above were used to continually reduce the temperature one degree by one degree. The cloudy temperatures of the polymer solutions at different concentrations were recorded. Therefore, the curve of the cloudy point via PCL concentration was drawn.

Porous PCL scaffolding caused by phase separation

PCL solutions in dioxane or dioxane/water (90/10 v/v) with different concentrations were prepared; 2 mL of each PCL solution was pipetted into a Teflon cap, which was then immediately placed in a freezer maintained separately at the temperature of 2°C and -30°C for 10 hours to allow phase separation to occur. PCL gels formed during the above phase separation were then freeze-dried at -58°C for 2 days to remove the solvent of dioxane or dioxane/water and leave the PCL phase with porous structure. The samples were kept in a desiccator for further characterization.

PCL-PGLA composite tube fabrication

PCL dioxane solutions (5 mL) with concentrations varying from 8% and 15% PCL were prepared. PGLA tubes braided on the Teflon mandrel of 15 mm in length and 4 mm in diameter were vertically dipped into each of above solutions for 30 min. The tubes stuck with PCL solution were pulled out of the PCL solution at a constant speed, to ensure the tube was removed slowly from the solution in 5 sec. The above procedures were performed at room temperature, 28°C. Two sets of PCL coated tubes were quenched separately at -30°C in a freezer and 2°C in a refrigerator for 10 hours to let the PCL solution phase separate, which were then freeze-dried at -58°C for 2 days to remove the solvents. Therefore, the prepared PCL-PGLA composite tube scaffolds were kept in a desiccator for further characterization.

PCL porous structure investments

The PCL porous structure was observed under scanning electromicroscopy (SEM) (JSM-5800LV, JEOL). The pore size of the scaffolds was quantified by placing the SEM images under a CCD camera with a micro viewer and then analyzing the image captured on the computer monitor using a Quantimet 520 image analyzer and corresponding software.

Fibroblast cell seeding and culturing on tubular scaffolds

PCL-PGLA composite tube scaffolds 5 mm in length and with an inner diameter of 4 mm were chosen for cell seeding to test the biocompatibility. Three scaffolds were sterilized by ethanol. 3T3 mouse skin fibroblast cells in DMEM medium containing 10% bovine serum, 1% penicillin and streptomycin, were used for seeding on the
tubular scaffold with $5 \times 10^5$ cells for each. The cell seeded tube scaffolds were put in 24-well culture dishes with 2 mL of media for each well, and then incubated inside a CO$_2$ incubator (WTB Binder, Tuttlingen, Germany) at 37°C. The medium was changed at every 3 days of culturing.

At the culturing time of 7 days, three scaffolds with cells were taken out of the incubator for further investigation. One sample was directly observed under optical microscopy: living cells on both the inner side and outer side were observed in the images. The second sample was dried in ethanol solutions with increasing concentrations and observed under SEM. The last samples were placed in Tissue Freezing Medium® (Jung) and frozen at -80°C in a microton (South London Electrical Equipment Co, Ltd, UK), where they were sectioned into slices, the slices were then stained with hematoxylin and eosin (HE) solution for H-E histology investigation. It was observed from the H-E stained slices that the cell nuclei were blue (hematoxylin) and the extracellular matrix was pink (eosin).

**RESULTS AND DISCUSSION**

**Tubular structure braiding**

Tubular PGLA scaffolds of different braiding angles were prepared through a braiding machine by varying the gear ratios (Fig. 1). A higher braiding angle gave a tighter braiding structure. Since all the 20 carriers were used, braiding with a 2/2 intersection-repeating pattern was formed. This is known as the regular, plain or standard braid. If only 10 carriers were used, a diamond braid would be formed. Regular braids have better structural integrity than diamond braids due to the greater frequency of intersections. From previous studies conducted on textile-braided composites, it was concluded that increasing the braiding angle (from 0-90 degrees) would increase the modulus in the transverse direction, while reducing the modulus in the longitudinal direction and vice versa (10). Therefore, a braiding angle was fixed at approximately 45-50 degrees resulting in a balance of the transverse and longitudinal properties of the braided tube. Figure 2 shows the SEM images of the braided tube. The gaps in between the braided yarns were about 200 µm in length, while those between strands were about 10 µm. But this braided structure looses easily if the Teflon mandrel support is pulled out of PGLA tube. That is the braided tube may collapse during cells seeding and culturing. Thus, PCL solution was used to coat on the PGLA braided tube to make a composite tube after the solvent was freeze-dried. Thus formed composite tube no longer collapse even without mandrel support. In addition, this coated PCL layer should also have a porous structure for cell proliferation.

**Porous coating by thermal induced phase separation**

Different methods have been reported for the preparation of porous scaffolds, such as particle leaching with different porogenous materials, like salt and paraffin (11, 12), gas forming (13), and thermal induced phase separation (TIPS) (14, 15). Considering that the residual porogenous materials remain a problem in the particle-leaching method special instruments are required for the
gas forming method. The TIPS method was chosen in this research for coating a porous layer on the surface of the PGLA braided tube.

The TIPS method for porous structure fabrication is based on the thermodynamic properties of the polymer, which has the upper critical solution temperature (Tc). At the temperature above Tc, the polymer is one phase solution state. If the temperature is lower than Tc polymer-rich and polymer-lean phases will form because of phase separation. After freeze-drying, the polymer-lean phase will form the pores and the polymer-rich phase will form the skeleton; therefore, a porous scaffold is prepared. The porous structure of the scaffold can be controlled just by a change in the polymer solution concentration, solvent properties, handling temperature as well as coarsening time. PGLA (15, 16), PLLA and its copolymer with PDLA (14, 17) have been investigated to prepare porous scaffolds using the TIPS method, but to date, there are no reports on the PCL solution and its thermodynamic behavior.

**Cloudy point of PCL solution**

We found in our experiments that PCL solution in dioxane will become clouded and finally phase separated if the temperature is reduced. Therefore, PCL solution in dioxane also has an upper critical solution temperature, even though there are no reports on the phase diagram of PCL dioxane solution. It reaches its cloudy point when the temperature is reduced. The cloudy point is defined as the temperature when phase separation of the previously homogenous solution is initiated and the solution appears cloudy. As the temperature was slowly lowered, the cloudy point for different concentrations could be recorded. Figure 3 shows the cloudy points of PCL in dioxane and dioxane/H2O. It was observed that the cloudy point increased with the increase in PCL concentration. The PCL solution in dioxane/H2O approaches the cloudy points at a higher temperature than that of the PCL dioxane solution; this means the addition of no solvent made the polymer solution phase separate at a relatively higher temperature.

**Effect of PCL concentration on pore structure and pore size**

Based on Figure 3, the cloudy point of the PCL solution, two phases of a polymer-rich phase and a polymer-lean phase will occur, if the solution is set at the temperature below its cloudy point. The polymer-lean phase in the form of droplets will separate inside the polymer-rich phase during phase separation. These droplets then leave pores after the solvent has been freeze-dried.

Figure 4 shows the SEM micrographs of the porous structure resulting from the PCL dioxane solution after quenching at -30°C for 10 hours and then being freeze-dried. It was found that the porous structure and porosity varied with the polymer concentrations. At a low polymer concentration of 1% and 5%, the pore structures obtained were irregular and elongated; there were large variations in pore sizes. When the PCL concentration increased to 8%, relatively regular and uniform porous structures were
obtained. At a PCL concentration of 15%, an interconnected porous structure was observed, but the pore size decreased further. If the PCL concentration increased to 25%, both pore size and porosity decreased significantly. Table I lists the mean pore size dependent on the PCL concentration, which were reduced from 30 to 3 µm when the PCL concentration increased from 1-25%. As our application was that of cell seeding and proliferation, the intermediate concentrations of 8% and 15%, which can give a pore size of about 10-25 µm, were chosen for the porous coating of the tube scaffold.

**Table I - Mean Pore Size of PCL Dioxane Casting Membrane by Thermally-Induced Phase Separation at Different Concentrations**

<table>
<thead>
<tr>
<th>PCL concentration in dioxane (w/v%)</th>
<th>Mean pore size (µm)</th>
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<tbody>
<tr>
<td>1%</td>
<td>30.2</td>
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<tr>
<td>5%</td>
<td>25.3</td>
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<tr>
<td>8%</td>
<td>13.6</td>
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<tr>
<td>15%</td>
<td>9.5</td>
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<tr>
<td>25%</td>
<td>3.3</td>
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**Effect of cooling rate on pore morphology and pore size**

PCL dioxane solution 8% and dioxin/water solution were separately quenched in a refrigerator at 2°C and then in a freezer at -30°C for the investigation of the cooling rate on pore morphology and pore size. The cooling rate, quenching in a refrigerator at 2°C, was slower than that in

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Fig. 4 - SEM micrographs of the PCL porous scaffold quenched at -30°C with different PCL dioxane concentrations, (a) 1%, (b) 5%, (c) 8%, (d) 15%, and (e) 25%.
The freeze-dried PCL solution obtained by varying the quenching temperature (a) and b) and the non-solvent (water) content ((A) and (B)). The slower cooling rate (with a quenching temperature of 2°C (b)), produced larger pores than the faster cooling rate (with a quenching temperature of -30°C (a)) for both PCL solutions in dioxane (A) and dioxane/water (90:10) (B). By varying the cooling rate, the time scales from the initial phase separation to that of solidification of the PCL would be different in each case. At the slower cooling rate, a relatively longer time could be given for the initial phase separation, and the polymer-lean droplets were able to grow bigger through the diffusion of the solvent in between the polymer-lean phase until the PCL-rich phase solidified and formed hard walls to stop the droplets’ growth. As a result, bigger pores were formed. This is known as the coarsening effect (16, 18), which is a kinetic behavior due to the thermodynamic minimization of interfacial energy spontaneously. This effect can cause the concomitant variation in the number and the size of the pores, because each droplet possesses different amounts of interfacial energy and grows at varying rates, and only the droplets with enough interfacial energy are able to continue growing and even merge. At faster cooling rates, that is to quench the polymer solution at a relatively low temperature, the droplets have no time to merge; meanwhile, the PCL solidified walls formed quickly because of the lower temperature and higher viscosity; as a result, more homogenous pores with smaller pore sizes formed. In this case, the coarsening effect is greatly reduced.

Fig. 5 - Coating surface morphology of 8% PCL solution separately in dioxane (A), and dioxane/water (90:10) (B), at the quenching temperature of a) -30°C and b) 2°C.
Effects of non-solvent on pore morphology and pore size

It can be seen from Figure 5 that the addition of no solvent in the PCL dioxane solution generated bigger pore size (B) compared with the PCL pure dioxane solution (A) regardless at what temperature the system was quenched. This result can be explained based on the cloudy point curve in Figure 3. The addition of water to the PCL dioxane solution has the effect of raising the cloudy point temperature at various PCL concentrations. Water, which is not the solvent of PCL, induces a weaker PCL and dioxane interaction. When cooling PCL dioxane/water solution to low temperatures, the phase separation will occur early at a relatively higher temperature, where the polymer solution still has lower viscosity and the polymer-lean droplets can merge into bigger droplets because of the tendency to minimize interfacial energy and the solvent diffusion in between; therefore, a bigger pore is formed after freeze-drying the solvent as in Figure 5 (B). With the increase in pore size, the number of pores decreased correspondingly and the pore interconnectivity decreased as well.

Cell growth on scaffolds

With processing parameters of 8%, PCL dioxane solution and a quenching temperature of -30°C, a PCL-PGLA composite tube was prepared as in Figure 6, which is an image of a braided PGLA (green color) tube coated with a PCL porous layer (white color) produced by the TIPS method. This tubular scaffold was chosen for fibroblast cell culture. Figure 7 gives the optical microscopy images of cell attachment and proliferation on the tube scaffold of the inner layer (a) and the outer layer (b) on day 7.
growth on the inner (a) and outer (b) surfaces of the tube scaffold at day 7 of culturing. Healthy cell debris was attached to both surfaces. Figure 8 shows the SEM micrographs of the composite tube section after cell seeding and culturing for 7 days. The cells covered both the inner and the outer side, but the PGLA fiber orientation can still be seen from the inner side. The wall thickness of this composite tube is about 500 µm.

To clarify whether the cells penetrated from the surface of the scaffold into the porous coating layer, the cross section of the cell-scaffold tube (after culturing for 7 days) was sliced and stained with hematoxylin and eosin. Figure 9 shows the H-E histology image taken at a magnification of x10 (a) and x20 (b) separately. As the cytoplasm of the cells would be stained red, the reddish region on the outer surface of the scaffold indicated a layer of cells attached, that was in agreement with what was previously observed under the microscope in Figure 7. The purplish dots and lines separated in the porous region indicated that the fibroblast cells had penetrated to the inside of the coating layer after culturing for 7 days. Therefore, the PCL coating layer was porous enough to allow cell penetration.

CONCLUSION

A biodegradable composite tube with porous PCL embedded and coated on a PGLA braided tube was fabricated for use as a blood vessel tissue engineering scaffold. A PCL porous structure was produced by thermal induced phase separation. The pore morphology and pore

![Fig. 8 - SEM micrograph of the PCL-PGLA composite tube with fibroblast cells cultured for 7 days.](image)

![Fig. 9 - H-E histology of the tube scaffold with fibroblast cell growth on the PCL porous coating surface, with a magnification x10 (a) and x20 (b). Cells penetrated into the inner porous surface of the PCL porous coating.](image)
sizes were determined by changing the polymer concentration, solvent composition and system quenching temperature. The addition of no water solvent made the polymer solution phase separate at a higher temperature and induced bigger pore sizes in the PCL scaffold. The coarsening effect became bigger if the polymer system was quenched at 2°C than at -30°C, and induced bigger pore size. The mean pore size decreased from 30 to 3 µm with the increase in polymer concentration from 1-25%, when quenching the PCL dioxane solution at -30°C. Therefore, PCL dioxane solutions with polymer concentration from 8-15% were suitable for PCL scaffolding, which resulted in uniformed pores a little bigger than the cell size of 10 µm. Fibroblast cells were successfully seeded on the PCL-PGLA composite tube. They grew well on the inner and outer surfaces, and had penetrated inside the PCL inner pores. The composite tube fabrication method reported in this paper has great potential to be developed for blood vessel tissue engineering scaffolding.

ACKNOWLEDGEMENTS

This research was supported by DAAD, Germany and Shanghai Sci. & Tech. Committee, China under Grant 05DJ14006.

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