3D fabrication of PCL micro-scaffolds with interconnected flow-channel and perfusion culture for *in vitro* construction of functional islet tissue

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***Abstract*—A novel micro-scaffold with interconnected flow-channel facilitating mass transfer was designed as a rigid structure to sustain dynamic perfusion culture towards engineering functional islet tissues. Biodegradable polycaprolactone (PCL) micro-scaffolds, having around 69.2% porosity with a pore size of 10 μm, were fabricated via casting technology using polyvinyl alcohol (PVA) mold printed by fused deposition modeling (FDM). Combined with perfusing or rotating dynamic culture, cells inside the scaffolds presented a high survival rate and the tendency of aerobic respiration, proving good biocompatibility characteristics and sufficient oxygen exchange. Enhanced glucose-stimulated insulin secretion of MIN6-m9 cells inside the scaffolds compared to the 2D model also indicated stronger cell activity and functions, providing an ideal candidate for *in vitro* tissue regeneration in the future.**

Keywords- Component; Micro-scaffold; Polycaprolactone; Perfusion Culture; Islets-tissue

I. INTRODUCTION

Diabetes is now one of the most common chronic diseases in the world, putting a heavy burden on people's health. Currently, subcutaneous insulin injection is the most commonly used treatment for diabetes[1]. However, long-term injection of insulin, patients need to not only endure the pain, but also bring down the drawbacks such as abdominal swelling, insulin allergy[2]. Therefore, it is necessary to develop effective diabetes therapy. With the rapid development of biomedical engineering, it has become feasible to fabricate transplantable functional islet tissue *in vitro* using tissue engineering strategies[3]. In addition to clinical transplantation, *in vitro* functional islet tissue can also be used in drug screening and islet cell biology research[4,5].

Focusing on the field of *in vitro* three-dimensional (3D) islet model in tissue engineering research, we investigated the existing *in vitro* functional islet micro-tissue technology and combined 3D printing technology to manufacture in vitro micro-scaffolds and used cell perfusion culture technology to build a three-dimensional system of substance transport functions, further obtaining functional islet tissue by cell culture[6-8]. The main results of this paper are: designed a micro-scaffold model with a good mass transfer function; explored the process flow of high-porosity three-dimensional scaffolds made by PCL, and optimized the parameters such as material solution concentration and freeze-drying temperature. Based on this, porous PCL scaffolds with internal flow channels and good connectivity were prepared. The surface treatment and protein coating process of bio-scaffolds were optimized, and islets with good activity were constructed using scaffolds. Perfusion was performed by using the three-dimensional islet model constructed by the PCL scaffold,

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which verified that the perfused three-dimensional islet model showed stronger functionality than the two-dimensional planar cultured islet cells. Our research provided new ideas for future diabetes cell therapy and construction of islet tissue drug screening models.

II. MATERIALS AND METHODS

A. Design And Fabrication Of The PCL Micro-Scaffold

The concept of our design is shown in our previous report[9], which had a Raschig-ring structure with a vertical hollow channel in the center. As shown in figure 1A, this micro-scaffold was 2000 μm in outer diameter, 800 μm in internal diameter, 2000 μm in height, and the diameter of the hollow channel which 700 μm from the bottom was optimized to be 600 μm. Firstly, the mold drawing of micro-scaffold was drawn by SolidWorks software (Soliworks 2019, Dassault Systemes, Paris, France), then the mold drawing was sliced by ideaMaker software (ideaMaker v 3.5.2, Raise3D, Shanghai, China) to get G-code file which can be used for 3D printing. PVA, a frequently-used printing material with high water solubility, was selected to print the support mold, and the mold as shown in figure 1B was obtained. PCL was dissolved in organic solvent 1,4-dioxane according to 15% mass solubility, then stirred and dissolved with an electromagnetic rotor at 800 rpm to obtain uniform PCL solution. The PCL solution was slowly injected into the pre-printed PVA mold with 1 ml syringe, and was respectively placed at - 4 °C, - 20 °C and - 80 °C for further solidification followed by lyophilization in an Alpha 1-2 freeze dryer (Martin Christ GmbH, Osterode am Harz, Germany) for 48 hours. 1,4-dioxane was sublimated during freeze-drying creating a porous structure inside the micro-scaffold.

After freeze-drying, the whole structure was placed in ultra-pure water for ultrasonic elution, and water was changed every 2 hours to remove water-soluble PVA mold. After 6 hours, the mold basically fell off. Then put the micro-scaffold in the beaker, and soak it with ultrapure water, use a magnetic stirrer to speed up the cleaning process, change the water every 12 hours. After 24 hours, the PVA mold was basically completely cleaned, and a porous micro-scaffold with internal cross-flow channels was obtained.

B. Scanning Electron Microscope (SEM) Observation And Porosity Measurement

Micro-scaffolds with different treatment were coated with gold-palladium (50 nm) and observed using a scanning electron microscope (SEM, FEI Quanta 200, Czech Republic) for morphological evaluation. The porosity of the micro-scaffold was measured by the quality of a single scaffold. The porosity ε is equal to 1 minus the filling factor F of the material as

$$\varepsilon=1-F \quad (1)$$

The filling factor F can be calculated by dividing the mass Mscaffold of the micro-scaffold by the mass of PCL with the same volume as

$$F=Mscaffold/Vscaffold\times\rho PCL \quad (2)$$

C. Cell Inoculation And Rotary/Perfusion Culture

MIN6-m9, a murine pancreatic β cell, was a concentration-dependent glucose-responsive cell line provided by People's Hospital of Peking University. The cells were cultured in high-glucose Dulbecco's modified Eagle medium (H-DMEM; Invitrogen) with 15% fetal bovine serum (FBS; Gibco), 1% penicillin and streptomycin (BI), and 50 μmol/L mercaptoethanol (M8210-100; solarbio) in a 5% CO_2 incubator at 37 °C.

Before inoculation, the micro-scaffolds need to be sterilized treatment and protein coating. First, the eluted PCL micro-scaffolds were placed in a 6-well plate, soaked in 70% ethanol solution for half an hour and placed in a clean bench with UV sterilization turned on. After half an hour, suck up the ethanol solution and continue to irradiate for another half an hour. Then, the 10% sodium hydroxide solution was soaked for half an hour for alkaline hydrolysis to improve the hydrophilicity of the scaffold. Then soak in a 3% hydrochloric acid solution for 3 minutes to neutralization.

After sterilization and surface treatment, the scaffold was cleaned with sterile water three times. To improve the cell adhesion rate and the biocompatibility of the micro-scaffold surface, the scaffold was immersed in a laminin solution (5 μg/ml in Hank's balanced salt solution) and vacuumed for 1h to ensure that the solution enters the internal pores. And incubation in a 37 °C incubator for 2 hours, then wash the micro-scaffold 3 times with HBSS to clean the excess laminin.

Around 40 laminin-coated micro-scaffold were placed in one well of a non-treated 6-well plate, which contained MIN-6 cell suspension (6.45×10^6 cells in 2.5 ml culture medium). After rotating on a shaker at 75 rpm for overnight inoculation, free cells were removed by gently washing with PBS. The rotating speed of the shaking was 85 rpm during rotary culture. The conditions of bioreactor and perfusion culture are consistent with our previous reports[9].

D. Measurement Of DNA Content And Live/Dead Assay

Cellular DNA content in the micro-scaffold were detected by 4',6-diamidino-2-phenylindole (DAPI) fluorometry (C1005; Beyotime). First, prepare a solution of known DNA concentration (0, 6, 12, 24, 48, 96 μg/ml) for obtaining the standard curve of reference calibration. Second, different concentrations of cell suspension (0.3125, 0.625, 1.25, 2.5, 5, 10×10^6 cells/ml) were obtained by mixing different numbers of MIN-6 cells in monolayers with PBS and sonicated at 40W for 1 minute in an ice bath. Then, DAPI solution (0.1 μg/ml) was mixed with the cell sample in the proportion of 60:1 and measured by a fluorescence spectrometer (HORIB-FLUOROMAX-4; HORIBA JOBIN YVON), then standard curve was obtained. Cell-loaded PCL/PLLA micro-scaffold were cut into small pieces, then sonicated and centrifugated at 600 rpm for 5 minutes. Finally, the DNA content of the sample was measured and calculated according to the standard curve in the second step.

Cell survival live/dead staining of the cell-loaded micro-scaffolds was collected on days 1 and 7. Briefly, the samples were washed thrice with PBS, followed by incubating

with the mixed dye solution of 1 μM calcein-am (Sigma; 17783) and 2 μM propidium iodide (Sigma; P4170) for 15 min at room temperature in the dark. Then images were captured by a laser scanning confocal microscope (LSCM, Nikon, Z1). Cell viability was calculated by counting the number of cells in Image-J ((live cell/total cell)×100%).

E. Analysis Of Glucose Consumption, Lactate Produce And Insulin Secretion

The supernatants of the samples were collected at various time points for the analysis. Glucose concentration was quantitatively detected by a biochemistry analyzer (YSI 2950, YSI Life science). Lactate produce (Sigma; MAK064) was measured using a corresponding determination kit following the manufacturer's instructions.

Glucose-stimulated insulin secretion assay was taken to analyze the activity and function of the 3D islet constructs cultured by perfusion. Briefly, perfused islet constructs were harvested on day 7 and bathed into a glucose-free medium for 30 minutes, followed by cultured with high glucose medium. Insulin secretion was sampled at specific intervals within 120 minutes and quantified by ELISA Kit (Abebio; AE38004MO). The insulin secretion of MIN-6 cells on flat culture dishes was adapted as the control group.

F. Statistical analysis.

All data are presented as the mean ± s.d. Paired two-tailed Student's *t*-tests was performed to compare two groups. We considered differences to be statistically significant when $P < 0.05$.

III. RESULTS AND DISCUSSION

A. PCL micro scaffold and culturing system

PCL micro scaffolds with channels inside were designed and fabricated. Under the optimal process parameters, PVA stacked support scaffolds were constructed as designed (figure 1B). PCL scaffolds with a ring shape were constructed using casting and dissolving process to achieve a size of 2000 μm × 2000 μm × 2000 μm with internal channels in a diameter of 600 μm (figure 1B). The channels would facilitate liquid flow and nutrition exchange in rotating (figure 1C) or perfusing (figure 1D) based dynamic culture. The design and construction of the packed dynamic bioreactor were shown in figure 1E.

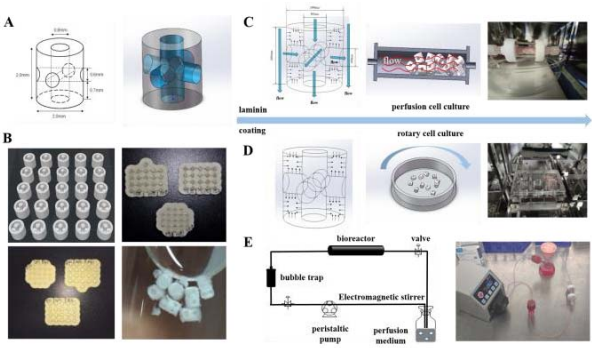

Figure 1. (A-B) The design and fabrication of PCL micro-scaffolds. (C) Perfusion cell culture system. (D) Rotary cell culture system. (E) Packed bioreactor.

B. Microstructure and porosity

PLA micro scaffolds obtained were shown in figure 2A, illustrating the cylindrical shape and intersecting internal flow channels. The SEM pictures also illustrated different microstructure caused by varied prefrozen temperature for freeze-drying (figure 2B). No clear micropores formed at - 4 ℃. When prefrozen at - 20 ℃, micro scaffolds containing evenly distributed 10 μm micropores were constructed, with calculated porosity as 69.2%. With prefrozen temperature further dropping to - 80 ℃, the size of a majority of micropores shrank to 2 – 4 μm, leading to a more stable structure.

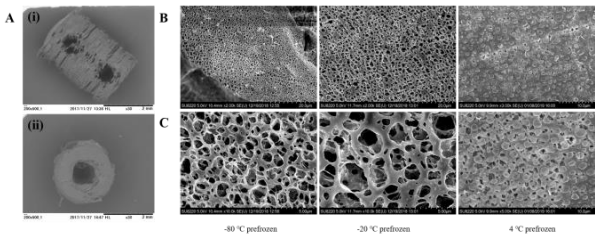

Figure 2. SEM images of PCL micro-scaffolds. (A) i: Side view of the scaffold. ii: Top view of the scaffold. Scale bar, 2mm. (B) Pore distribution with the prefrozen temperature of -80 °C, -20 °C, 4 °C respectively. Scale bar, 10/20μm. (C) High magnification view of (B). Scale bar, 5/10μm.

C. Biocompatibility test.

PCL scaffolds just after cell inoculation were shown in figure 3A, revealing the complete structure and clear internal flow channels. Live/dead staining pictures on day 1 proved that a large number of MIN-6 cells were attached to the laminin treated scaffolds with a high survival rate (Figure 3B). On day 7, the cell survival rate increased to 74.5±8.7% from 66.6±5.5% on day 1. Meanwhile, the number of cells loaded onto or inside the scaffolds both showed an increasing trend, no matter under the dynamic conditions of rotating (Figure 3C) or perfusing (Figure 3D). However, there are differences in cell loading capacity between these two dynamic methods. On day 7, the cellular DNA content of perfused scaffolds was around 2.5 folds that of rotating ones (Figure 3E). The difference in cell numbers on the surface could also be directly illustrated by fluorescent staining results (Figure 3C, D). The large number of cells shed from the surface of rotating scaffolds may due to

the excessive shear force caused by violent rotation. Cells on the surface bear much less shear force during perfusing culture, so they can proliferate and grow stably on scaffolds.

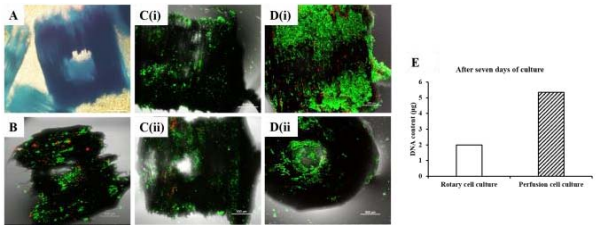

Figure 3. (A) Image of PCL micro-scaffold after cell inoculation. Cell viability after MIN-6 inoculation in PCL micro-scaffold on day 1 (B) and day 7 of cultivation with the rotary system (C) or perfusion system (D), where live cells are stained in green and dead cells in red. Scale bar, 500 μm. (C-D) i: Crosscutting view of PCL micro-scaffold. ii: Top view of the scaffold. (E) DNA content on each scaffold after 7 days of rotary culture or perfusion culture.

D. Metabolic features

Glucose is the energy source of cell metabolism in scaffolds. When oxygen is sufficient, glucose will be completely oxidized and decomposed by aerobic respiration to carbon dioxide and water. If not, lactate will be secreted into the culture medium as a byproduct of anaerobic respiration. Thus, the fluctuation of glucose consumption and lactate production over time would reflect the metabolic features of cells, further evaluating the oxygen supply to cells inside the scaffolds. The results for two dynamic methods on day 2, 4, and 7 were shown in figure 4. The results revealed that glucose consumption increased with days for both methods, which was caused by cell proliferation. While, the consumption quantity and growth rate of perfusing were both significantly higher than those of rotating, which was due to the higher attached cell number and also consistent with the DNA content and fluorescent results above. The lactate production in the rotating culture was 51.2, 71.3, and 59.9 mg/day per 106 cells on day 2, 4 and 7 respectively, a stabilized tendency proving a sufficient oxygen supply to cells. There was a higher lactate production in the early stage of perfusing as 152.2 mg/day per 106 cells on day 2, which might be related to the excessive adhered cell number initially and the lag of response. With the increase of culture days, lactate production decreased significantly to 91.6 mg/day per 106 cells on day 4, indicating an ensured oxygen supply even accompanied with cell proliferation. The analysis of metabolic features proved that the design of the micro scaffold and the dynamic culture co-guaranteed a sufficient exchange of nutrients and oxygen.

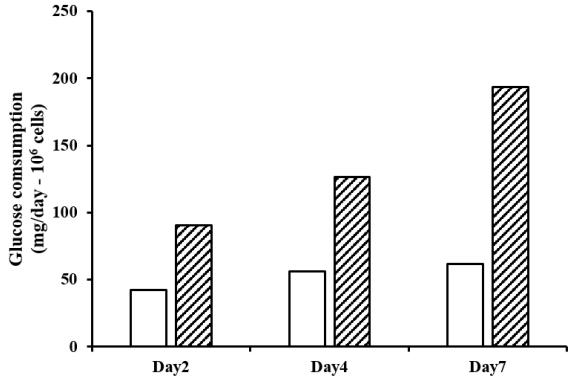

Figure 4. Glucose consumption of MIN-6 cells in PCL micro-scaffold with rotary culture or perfusion culture. All data normalized to retained cell numbers in different PCL micro-scaffolds.

E. Insulin secretion of 3D structure

Glucose-stimulated insulin secretion of perfused 3D scaffolds and 2D control group within 120 minutes were shown in figure 5. Compared with the control group, cells in the 3D microenvironment of the scaffold presented stronger cell activity and function under the same cell number and stimulation conditions.

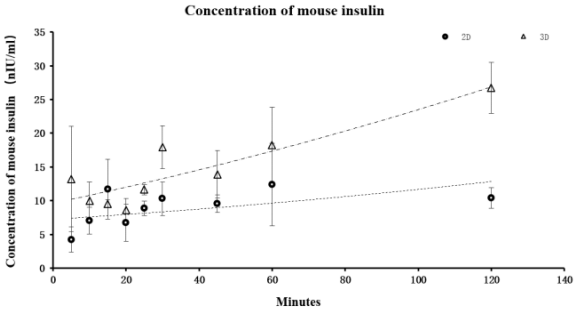

Figure 5. Glucose-stimulated insulin secretion of perfused 3D structure and 2D control group

IV. CONCLUSION

In this research, we developed a novel method for manufacturing PCL micro scaffolds with internal flow channels. A Raschig-ring structure was constructed using casting and dissolving PVA support mold to achieve a size of 2000 μm × 2000 μm × 2000 μm with internal through channels in a diameter of 600 μm. Scaffolds after freeze-drying contained evenly distributed 10 μm micropores and porosity as 69.2%. Cell survival rate and metabolic features both illustrated that the micro scaffolds have good biocompatibility characteristics and guaranteed exchange of oxygen when combined with perfusing or rotating dynamic culture. Glucose-stimulated insulin secretion also indicated stronger cell activity and functions in the scaffold compared with 2D models, providing an ideal candidate for *in vitro* tissue regeneration in the future.

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