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SHORT COMMUNICATION

Chitosan microfiber fabrication using a microfluidic chip and its application to cell cultures

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Abstract In this study, a poly-methyl-methacrylate (PMMA) microfluidic chip with a 45° cross-junction microchannel is fabricated using a CO_2 laser machine to generate chitosan microfibers. Chitosan solution and sodium tripolyphosphate (STPP) solution were injected into the cross-junction microchannel of the microfluidic chip. The laminar flow of the chitosan solution was generated by hydrodynamic focusing. The diameter of laminar flow, which ranged from 30 to 50 μm, was controlled by changing the ratio between chitosan solution and STPP solution flow rates in the PMMA microfluidic chip. The laminar flow of the chitosan solution was converted into chitosan microfibers with STPP solution via the cross-linking reaction; the diameter of chitosan microfibers was in the range of 50–200 μm. The chitosan microfibers were then coated with collagen for cell cultivation. The results show that the chitosan microfibers provide good growth conditions for cells. They could be used as a scaffold for cell cultures in tissue engineering applications. This novel method has advantages of ease of fabrication, simple and low-cost process.

Keywords Chitosan · Microfluidic · Microfiber · Cross-linking reaction

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1 Introduction

There are many researches on polymer material, including alginate, chitosan, PLGA, etc. They are also biodegradable (Onishi and Machida 1999) and biocompatible, which makes them suitable compounds for biomedical applications, such as wound management (Rao and Sharma 1997), tissue engineering (Peng et al. 2000) for nerve regeneration in clinical applications (Freier et al. 2005), and drug delivery (Mi et al. 2002; Roy et al. 1999; Ribud et al. 2000).

Recently, more and more implantable applications of chitosan have been reported (Khor and Lim 2003). In an acidic environment, the surface of chitosan binds to the positive group, which improves cell adhesion and growth (Madihally and Matthew 1999; Yuan et al. 2004). Chitosan microfiber has been fabricated for medical products. However, chitosan as a biofiber is dimensionally unstable and has poor mechanical properties. The cross-linking reaction was used to enhance the structure and fix the shape by chemical reagents. The epoxy compounds, glutaraldehyde and formaldehyde can be used for chitosan cross-linking reaction. But they could remain toxic in the organism and cause damage. The sodium tripolyphosphate (STPP) for cross-linking reaction of the chitosan was reported (Shiraishi et al. 1993). STPP is a non-toxic biomaterial and has a stable cross-linking reaction with chitosan (Mi et al. 1999; Leea et al. 2002).

The microfluidic techniques have provided a facile approach for the synthesis and fabrication of monodisperse polymer particles in the micrometer size range (Kim et al. 2007; Zourob et al. 2006; Oh et al. 2006; Jeong et al. 2005; De Geest et al. 2005; Quevedo et al. 2005; Nie et al. 2005). The rapid development of microfluidics has led to a considerable variety of microfluidic devices. Recently, a

number of methods are available by utilizing the microfluidics to synthesize shape-controlled microparticles. For example, Lin et al. used microchannel cross-junction (Huang et al. 2006, 2007; Yeh and Lin 2009), Zhang et al. (2006, 2007) used the Y-shape microfluidic devices, Sugiura et al. (2005) and Kobayashi et al. (2005) used a micronozzle array, and Liu et al. (2003) used membrane emulsification technique for the particles.

The aim of this study is to develop a new method for generating the chitosan microfibers to be scaffolds and apply them for culture cells. In our study, the cross-junction (45° of angle) was designed for the microfluidic chip. By changing the different ratio of the core and sheath flow rate, different sizes of chitosan microfibers can be produced. And then schwann cells and fibroblast cells were cultured in the chitosan micorfibers to be the scaffold in the tissue engineering.

We use the laser ablation to fabricate the microfluidic chip by CO_2 laser machine, fabrication process of which is simpler, lower cost, and quicker than casting molding, imprinting, and injection molding. The material of the microfluidic chip is poly-methyl-methacrylate (PMMA), which is the common polymer to fabricate the chip. Another common polymer is PDMS, but it requires the soft-lithography technology and casting molding to fabricate the microfluidic chip, which makes the method complicated, time wasting, and high cost. Thus, we adopted the simple method (laser ablation on PMMA) to fabricate the chip and used the chip to produce the chitosan microfibers.

2 Materials and methods

2.1 Materials

Chitosan was obtained from Sigma Chemical Co. (USA) and dissolved in 2% HAc solution. The degree of deacetylation was 85% and Mv was 600,000. Sodium sulfate, sodium citrate, and STPP were obtained from Sigma Chemical Co. (USA). All other reagents used were of reagent grade.

2.2 Simulation of the microfluidic chip

CFDRC-ACE software was used to simulate the diameter variation of laminar flow in the microfluidic chip. Three modules were used to simulate the chip: flow, free surfaces, and the grid deformation phase. The core flow had a velocity of 2.672×10^{-3} m/s, a density of 997 kg/m^3, and a viscosity of 0.001 kg/ms. The sheath flow had a velocity of 2.689×10^{-3} m/s to 2.905×10^{-3} m/s, a density of 917 kg/m^3, and a viscosity of 0.028 kg/ms in the microchannel. The 45° cross-junction microchannel had a width of 200 μm. The density distribution showed various diameter sizes of laminar flow for different flow rate conditions.

2.3 Design and fabrication of the microfluidic chip

AutoCAD® 2008 (Autodesk, USA) was used to design the developed microfluidic chip. The design was laid out on a conventional PMMA substrate (length/width/depth: 270 mm/210 mm/1.5 mm) with a laser micromachining process using a CO_2 laser machine (LaserPro Venus, GCC, Taiwan). The microfluidic chip had three layers, (as shown in Fig. 1a), the cover layer, the main layer, and the bottom layer. The layers were bound by screws (tightened at 1–1.6 Nm), as shown in Fig. 1b. The microfluidic chip has three inlet ports, one cross-channel (angle of 45°), an observation chamber, and one outlet. The channel width size of the chip was about 200 μm, the depth of the chip was 1.5 mm and curve channel part was 600 μm.

2.4 Principle of chitosan micorfiber generation and experimental procedure

In this study, we report the use of microfluidics to control the spontaneous self-assembly of microfibers using a solution of dissolved chitosan. The sheath force at the cross-junction microchannel forms a narrow size distribution of self-assembling laminar flow structures, the so-called laminar flow of the chitosan solution. When these laminar flows interact with the STPP solution at the 45°cross-junction position, the chitosan laminar flow reacts with $P_3O_{10}^{5-}$ ions. After ionic-cross-linking, STPP-chitosan microfibers can be observed.

The experiment procedure is as follows. The fluids of the center and side channels are set up with 0.5% chitosan solution (core flow) and STPP solution (sheath flow), respectively. The fluids are then injected into the microfluidic chip by syringe pumps (KD Scientific KDS230) programmed by a PC. In this work, the hydrodynamics

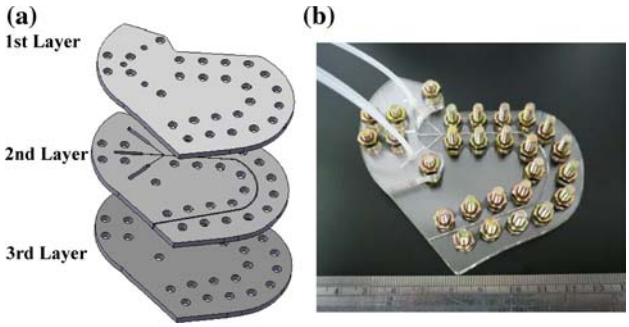

Fig. 1 **a** Illustration of microfluidic chip and **b** photograph of the microfluidic chip

were focused using a stream of aqueous chitosan solution at a 45° cross-junction microchannel by two STPP streams, enabling the construction of laminar flow of chitosan solution along the microchannel axis. The laminar flow of chitosan solution undergoes cross-linking with the STPP solution and $P_3O_{10}^{5-}$ ions to produce the chitosan microfibers. A scheme of the formation of STPP-chitosan microfibers is shown in Fig. 2.

2.5 The procedure of the culture cell on the microfibers

We cultured the schwann cells and fibroblast cells in the chitosan microfibers to use in tissue engineering. The chitosan microfibers were washed in PBS solution, and then soaked in the PBS solution to adjust the pH value to about 7. Then, the chitosan microfibers were placed in a non-culture dish, and sterilized using UV light. After 24 h, the surface of chitosan microfibers was coated with collagen to promote cell adhesion. The cells adhered to the chitosan microfibers, which were then washed in PBS solution to remove unadhered cells. After culturing the cells at 24, 48, 72 h respectively, we observed the growth of cells on the chitosan microfibers.

2.6 Observation and measurement of microfiber size

An optical microscope (BX60, Olympus, Japan) and SEM (Field-Emission Scanning Electron Microscope/JEOL JSM-7000) were used to observe the microfibers. The diameters of each laminar flow and the microfibers were measured using a digital camera (DP70, Olympus, Japan).

3 Results and discussion

3.1 Simulation of the microfluidic chip

In the simulation, hydrodynamic focusing and laminar flow were generated at the cross-junction microchannel, as shown in Fig. 3. Using the density distribution, we could observe the various diameters of laminar flow. Under a core flow of 2.672×10^{-3} m/s, the diameter size of laminar flow decreased when the sheath flow rate was reduced from 2.689×10^{-3} m/s to 2.905×10^{-3} m/s, as shown in Fig. 3a–d. Figure 3e shows the relationship between the sheath flow rate and the diameter size of laminar flow. The microfluidic chip with a 45° microchannel generated laminar flow. The diameter of the laminar flow was controlled by adjusting the sheath flow rate.

3.2 Formation of chitosan laminar flow

For the generation of continuous laminar flow, a pregel solution (25 mL of 0.5% (w/v) chitosan solution) and STPP solution were employed as the core phase and the sheath phase, respectively. The pregel solution was compressed by the sheath force in the microfluidic chip equipped with a 45° cross-junction channel, which resulted in continuous semi-products (chitosan microfibers).

In the experiments, we found that the chitosan solution was compressed by the STTP solution into an arrow shape, as shown in Fig. 4a. The laminar flow of the chitosan solution was about 40 μm in diameter when the chitosan solution was 0.5 mL/min and the STPP solution was

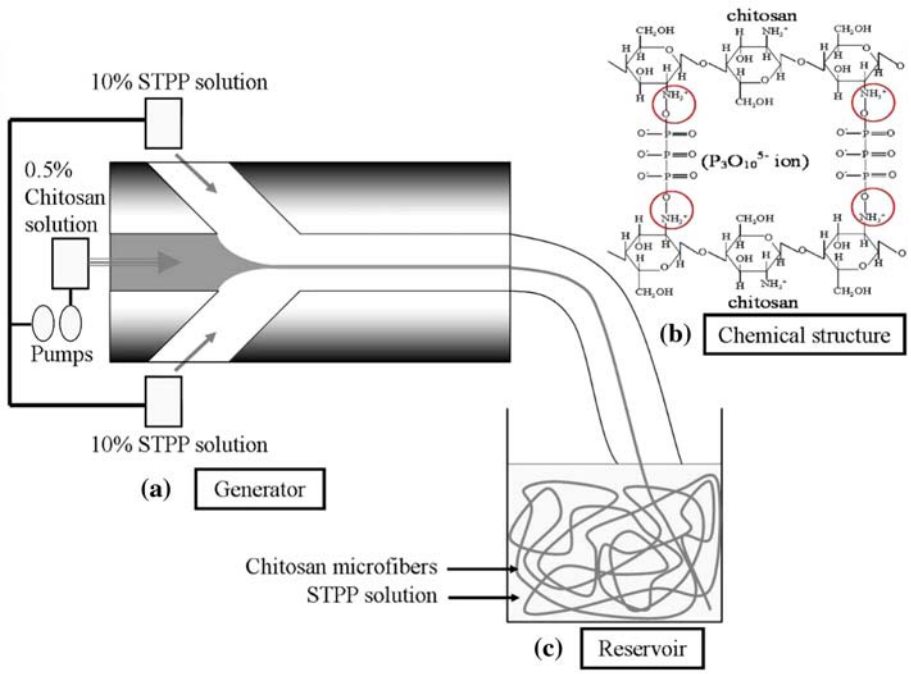

Fig. 2 Scheme of the STPP-chitosan microfiber generation system. Chitosan microfibers formed in the cross-junction microchannel. With 10% (w/v) TPP solution, the chitosan microfibers were transformed into STPP-chitosan microfibers in the chip

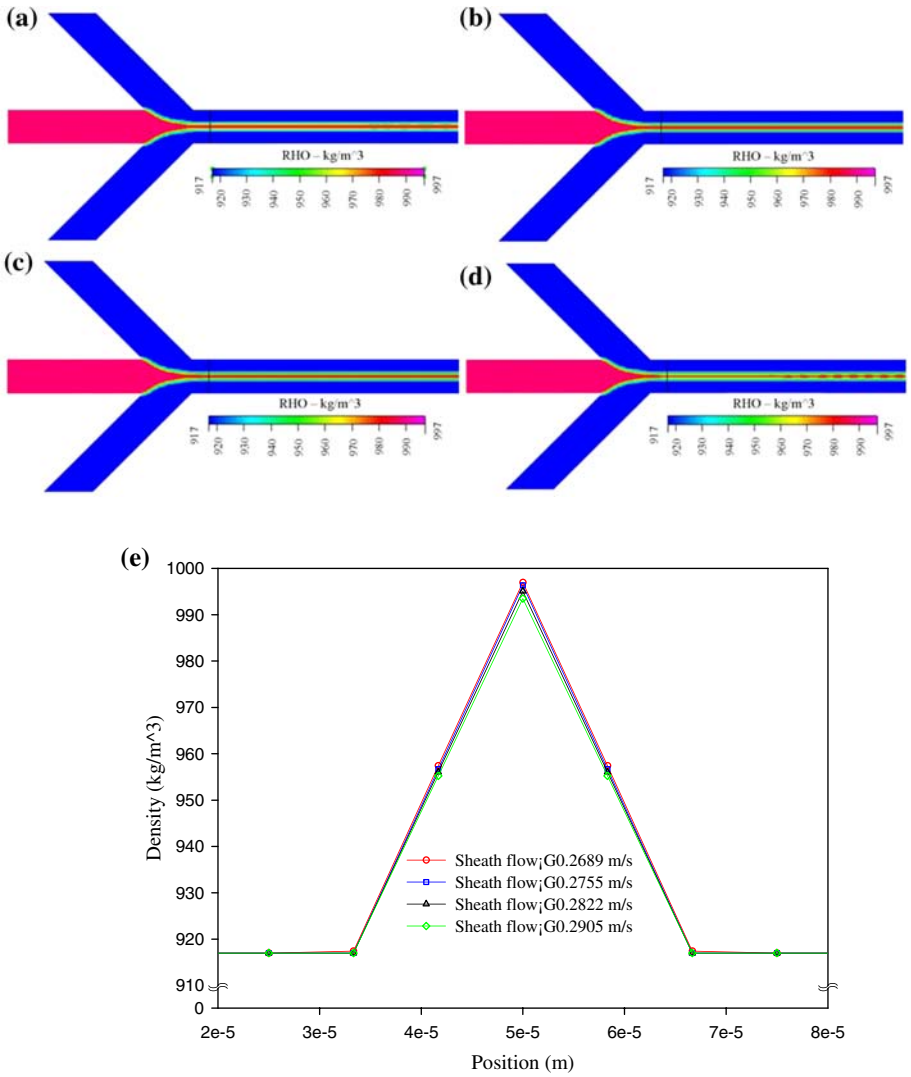

Fig. 3 **a–d** Simulation results of laminar flow generation, and **e** the simulation results of the relationship between the flow rate and diameter of laminar flow

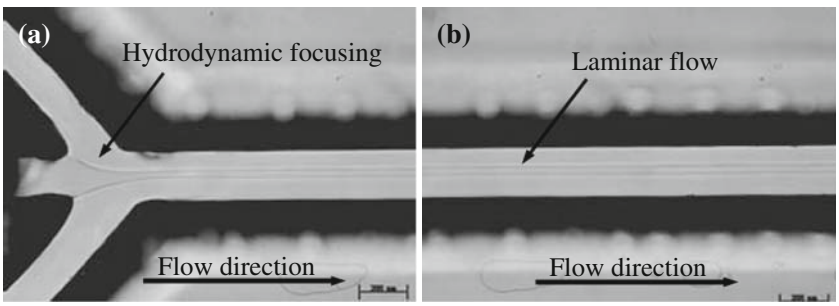

Fig. 4 Hydrodynamic focusing and gelation of chitosan laminar flow. Chitosan solution was hydrodynamically focused at the cross-junction (45°) of the channel (scale bar = 200 μm)

3.0 mL/min. The continuous laminar flow of the chitosan solution was generated by controlling the flow rates, as shown in Fig. 4b.

3.3 Formation of STPP-chitosan microfibers

The semi-products (chitosan microfibers) formed in the $P_3O_{10}^{5-}$ of 10% (w/v) STPP solution flow. After ionic-cross-linking, the laminar flow of chitosan was transformed into STPP-chitosan microfibers. Then, the STPP-chitosan microfibers were transported to the STPP solution reservoir. The microfibers were fully cross-linked. The chitosan microfibers were slightly dilated and hardened in 10% STPP solution. The diameter of the microfibers was about 50 μm, as shown in Fig. 5a, b. The fully cross-linked microfibers were strong and not aggregated

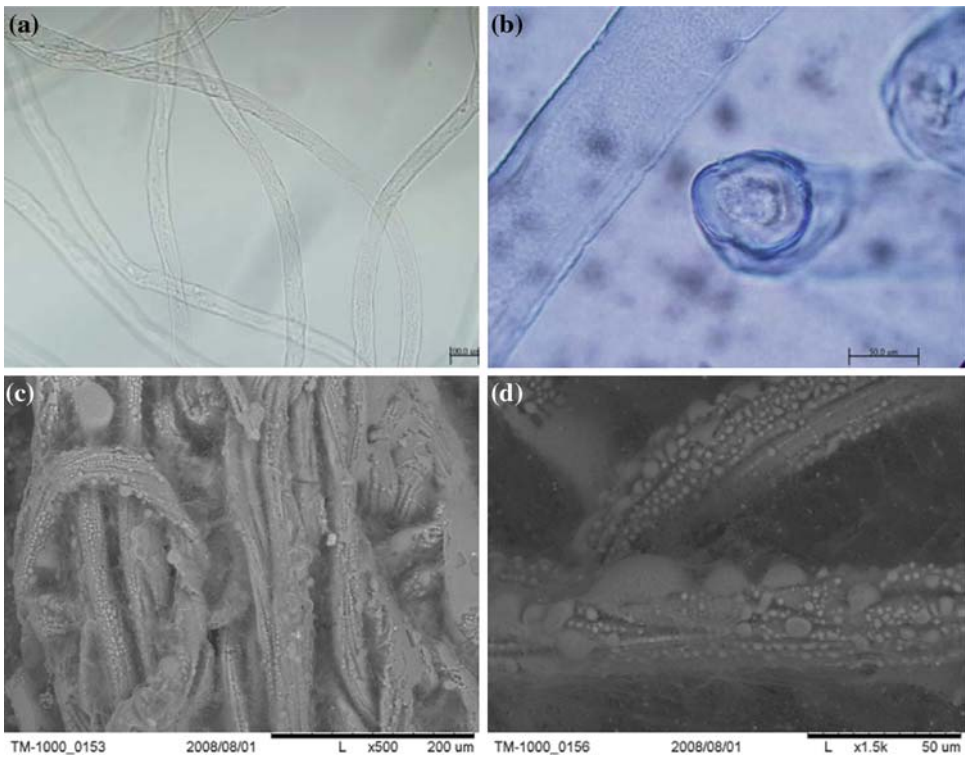

Fig. 5 Morphology of the prepared STPP-chitosan microfibers in the reservoir. **a** 20× Magnification microscope photographs of prepared STPP-chitosan microfibers, and **b** 50× magnification microscope photographs of STPP-chitosan prepared microfibers. SEM morphology of the STPP-chitosan microfibers at **c** 500× and **d** 1500×

with each other in the 10% STPP solution reservoir. SEM was used to observe the surface of the chitosan microfibers; the results are shown in Fig. 5c, d.

3.4 Influence of flow rate

The diameter of laminar flow was controlled by altering the ratio between chitosan solution and STPP solution flow rates in the microchannel. The diameter of chitosan laminar flow decreased from 50 to 30 μm when the core flow rate was decreased from 0.7 to 0.1 mL/min with a fixed sheath flow rate of 3.0 mL/min, as shown in Fig. 6a–d. Figure 6e shows the relationship between the flow rates of the two phases and the diameter of laminar flow. For a given 0.1 mL/min of core flow, the diameter of chitosan laminar flow decreases as the average velocity of the sheath flow increases. For a given 2.0 mL/min of sheath flow, the diameter of chitosan laminar flow increases as the average velocity of the core flow increases. The results show that the diameter of chitosan laminar flow, generated in the 45° cross-junction, can be controlled using the developed microfluidic chip.

3.5 The result of the cell culture with chitosan microfibers

In the cultured schwann cells with chitosan microfibers experiment, the schwann cells adhered on the surface of the

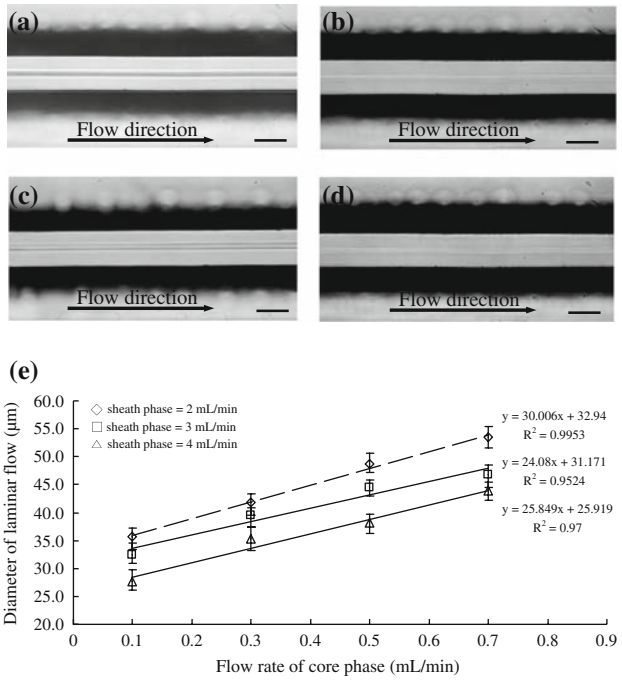

Fig. 6 Laminar flow diameter with STPP flow rate fixed at 3 mL/min for the core flow rate of **a** 0.7 mL/min, **b** 0.5 mL/min, **c** 0.3 mL/min, and **d** 0.1 mL/min. (scale bar = 200 μm). **e** The relationship between diameter of laminar flow and core flow rate

chitosan microfibers after 24 h, as shown in Fig. 7a. After 72 h, the schwann cells showed the high proliferation and grew along a straight line, as shown in Fig. 7b.

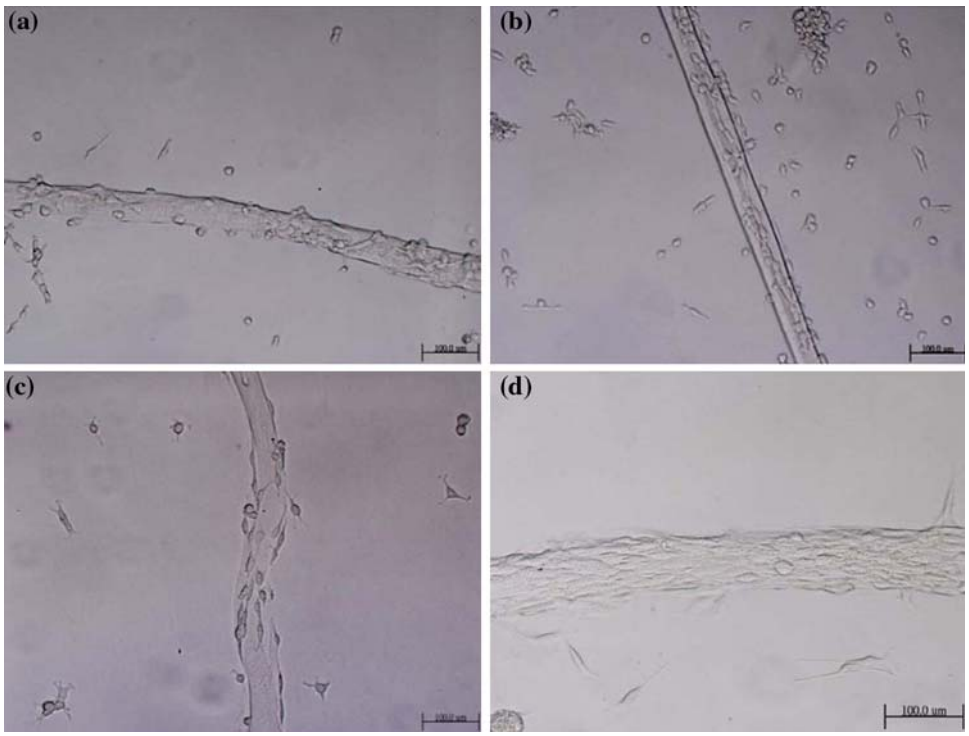

Fig. 7 Photographs of schwann cells with chitosan microfibers **a** after 24 h, and **b** after 72 h. Photographs of fibroflast cells with chitosan microfibers **c** after 24 h, and **d** after 72 h

In the cultured fibroblast cells with chitosan microfibers experiment, the fibroblast cells adhered on the surface of chitosan microfibers after 24 h, as shown in Fig. 7c. After 72 h, the fibroblast cells showed the high proliferation and covered the surface of the chitosan microfibers, as shown in Fig. 7d. These results demonstrate that the chitosan microfibers provide a good scaffold for cell cultures for tissue engineering applications.

4 Conclusions

In this study, we generated chitosan laminar flow using the sheath force in a microfluidic chip and produced STPP-chitosan microfibers via the ionic-cross-linking reaction. A 45° cross-junction microchannel was used to generate the chitosan microfibers. The laminar flows were generated by altering the core and sheath flow rates to obtain chitosan laminar flow diameters of 30 to 50 μm. The diameters of chitosan microfibers ranged from 50 to 200 μm after cross-linking. Schwann and fibroblast cells grew along the chitosan microfibers.

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