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Fiber-Based Chitosan Tubular Scaffolds for Soft Tissue Engineering: Fabrication and *in Vitro* Evaluation*

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Abstract: Porous, two-ply tubular chitosan conduits for guided tissue regeneration were fabricated by combining the textile technique (inner layer) with the thermally induced phase separation process (outer layer). A hollow chitosan tube was prepared using an industrial warp knitting process with chitosan yarns. Then, an appropriate diameter mandrel was inserted into the pre-fabricated tube. The tube and the mandrel were dipped into the chitosan solution together, taken out, and freeze-dried. After being neutralized in alkaline solution and dried at room temperature, the mandrel was removed to create the chitosan tubular scaffold. Scanning electron micrographs show that the resulting tubes have a biphasic wall structure, with a fibrous inner layer and a semipermeable outer layer. The swelling properties and the mechanical strength before and after *in vitro* degradation were investigated. The biocompatibility of the scaffolds was also investigated by co-culturing neuroblastoma cells (N2A, mouse) with the scaffolds. The results suggest that these chitosan tubular scaffolds are useful for the regeneration of tissues requiring a tubular scaffold.

Key words: nerve conduit; chitosan; knitting; tissue engineering

Introduction

Tissue engineering has emerged as a promising alternative for the treatment of malfunctioning or lost organs. These projects require different size tubular scaffolds as substrates and physical supports for organ and tissue regeneration to guide the formation of new organs, such as blood vessels, tracheas, and peripheral nerves.

Chitosan, a cationic natural biopolymer, is the *N*-deacetylated derivative of chitin, the most abundant natural polymer after cellulose. Chitosan is an interesting biomaterial due to its good biocompatibility, biodegradability, low toxicity, and low cost. Chitosan has already been used for drug delivery, sutures, and skin repair. In recent years, chitosan has also shown promise as a material for nerve regeneration, either used alone or blended with some other biomaterials[1-5]. Previous studies have shown that endothelial cells grow well on chitosan, which indicates the possibility of chitosan as an artificial vascular material[1]. But the technique of fabricating chitosan into tubular structures has always been a problem due to its inherent physicochemical properties. As a polysaccharide, chitosan will not melt or soften but instead carbonizes when heated. Therefore, chitosan tubes cannot be fabricated by commonly used heat processing methods. Chitosan also does not easily dissolve in common solvents and has only low solubility in some acidic

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solutions, making it impossible to manufacture chitosan scaffolds by foaming, salt-leaching, and three-dimensional printing methods. The authors' group has developed some methods for fabricating chitosan tubular scaffolds[6-9], for example, the circumvolving-vaporizing method and the freeze-drying method, but there are still some shortcomings including that the processes are very time-consuming, have large batch-to-batch variations, are unable to reliably fabricate long scaffolds, and the products are too rigid and brittle to be sutured in place.

The goal of this work is to establish an efficient method to produce porous chitosan tubular scaffolds on a large scale. This paper also reports the *in vitro* characteristics and properties of the scaffolds.

1 Materials and Methods

1.1 Materials

Chitosan (degree of deacetylation = 85.1%, viscosity-average relative molecular mass M_η=1.8×10^6) was purchased from Qingdao Haisheng Co. (China). Chitosan yarns (200 μm in diameter) were purchased from Qingdao Jifa Co. (China). Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, fetal bovine serum (FBS), and lysozyme were from Sigma Co. (USA). All other reagents were of analytical grade.

1.2 Preparation and morphology characterization of chitosan tubular scaffolds

Hollow chitosan tubes with various inner diameters and wall thicknesses were first prepared using an industrial warp knitting process. A segment of the knitted chitosan tube was cut off the strand with a proper size stainless steel mandrel then inserted into the pre-fabricated hollow tube. The tube was then dipped into 2% (*w/v*) chitosan acetic acid solution and rotated. After being taken out of the chitosan solution, the tube and the mandrel were positioned vertically and frozen at –20℃ for 12 h, and then dried in a freeze-dryer (ALPHA-1-4, Martin Christ Company, Germany). The freeze-dried samples were immersed into 2% (*w/v*) NaOH solution and equilibrated for 30 min to neutralize the acetic acid, then rinsed with distilled water several times until the rinsing solution was neutral, and finally dried at room temperature. The mandrel was removed to leave the chitosan tubular scaffold. A segment of the scaffold (5 mm in length) was coated with gold in vacuum and examined with a scanning electron microscope (SEM; KYKY-2000, China) at an accelerating voltage of 25 kV.

1.3 Swelling properties of porous chitosan tubular scaffolds

The inner and outer diameters of the samples were measured. Then the samples were immersed into 0.1 mol/L phosphate buffered saline (PBS, pH＝7.4) for 24 h at 37℃ to equilibrium. The dimensions of the hydrated samples were then measured again.

1.4 *In vitro* degradation

Chitosan tubular scaffolds (dried, 5 cm in length, with known mass) were immersed in 4 mg/mL lysozyme solution in 0.1 mol/L PBS (pH = 7.4) at 37℃. The degrading solution was replaced with fresh lysozyme solution every week. After a specified number of time intervals, the samples were taken out of the lysozyme solution and rinsed with distilled water. Some of the degraded samples were dried and weighed to determine the mass lost during degradation. Other samples were used to investigate the change of mechanical properties of the scaffolds. Both the compressive and tensile strengths were determined on a universal testing machine (AG-1, Shimadzu, Japan). The compressive strength was measured with a force perpendicular to the longitudinal axis of the scaffold at a cross-head speed of 1 mm/min. The loads corresponding to each transformation ratio were recorded at strains of 0.2, 0.4, and 0.6. The tensile strength was measured with the applied force parallel to the tube axis at an extension speed of 1 mm/min with an initial distance of 1 cm between two grips. The maximum stress during the extension was recorded.

1.5 Biocompatibility study

Neuroblastoma cells (N2A, mouse) were used as model cells to evaluate the biocompatibility of the scaffolds. The N2A cells were cultured in a medium of 47.5% DMEM and 47.5% OPTI-MEM with 5% FBS. The scaffolds were cut into 3-mm-thick slices that were then soaked in 75% ethanol for 24 h, rinsed three times in PBS, and then placed in a 24-well tissue culture cluster with 1×10^5 cells in each well. The

cultures were incubated at 37℃ in a humidified 5% (volume fraction) CO_2 incubator. The medium was changed every 2 days. After 5 days in the static culture, the scaffolds seeded with N2A cells were taken out, rinsed with PBS and fixed in 4% (mass fraction) glutaraldehyde solution. The samples were then dehydrated in graded ethanol and dried in an aeration cabinet before coating with gold for observation of the cell appearance both on the scaffold cross-section and on the fibrous inner layer with the SEM.

2 Results

2.1 Scaffold fabrication and morphology characterization

Porous, and tubular chitosan conduits were prepared using a warp knitting process in combination with the thermally induced phase separation technique. The scanning electron micrographs of the scaffolds show that the scaffolds had a biphasic wall structure, with a fibrous inner layer and a semipermeable outer layer (Fig. 1).

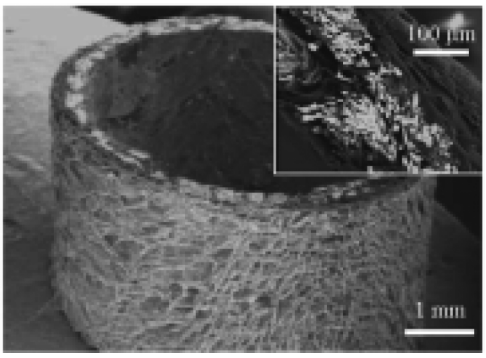

Fig. 1 Scanning electron micrographs of the chitosan tubular scaffold

2.2 Swelling properties of porous chitosan tubular scaffolds

The inner and outer diameters of the dry and hydrated samples are shown in Fig. 2. The data shows that the outer diameter of the hydrated scaffolds was significantly larger than that of the dry scaffolds (*p<0.05, N=6), while the inner diameter did not change much.

2.3 *In vitro* degradation

The mass and mechanical strength changes of the samples after being degraded in 4 mg/mL lysozyme *in vitro* are shown in Fig. 3 and Fig. 4. Figure 3 shows that the scaffold masses decreased during degradation, but the change was not significant, while the maximum stress of the samples during the tensile strength testing decreased significantly during degradation. After 6 months, the tensile strength of the scaffolds was very low. Figure 4 shows the relationship between the load and strain when a compressive force was applied perpendicular to the longitudinal axis of the scaffold. The load was significantly higher for the normal tubes than for the tubes degraded for 6 months at each strain, and just slightly higher than for the tubes degraded for 2 months.

2.4 Biocompatibility study

Figures 5 and 6 show the appearance of the N2A cells cultured on the scaffold for 5 days. The N2A cells migrated and flourished both on the cross-section and on the fibrous inner layer of the tube. The result indicates that the scaffolds have a very good cytocompatibility.

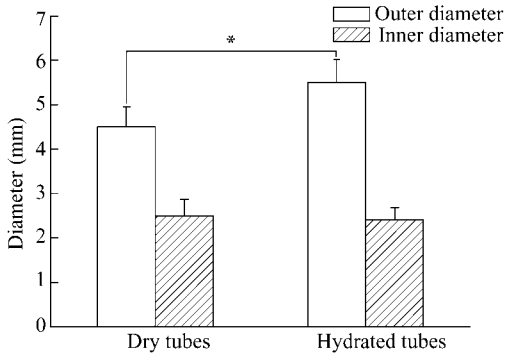

Fig. 2 Scaffold swelling (*p<0.05, N=6 in each group)

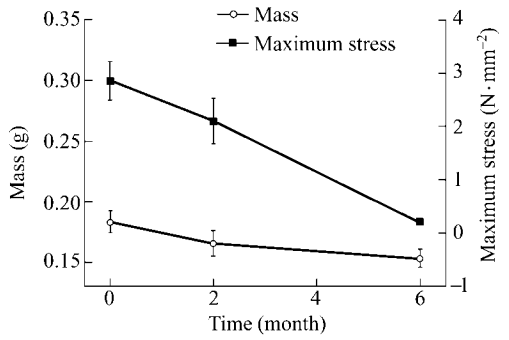

Fig. 3 Mass and maximum stress of the scaffolds during *in vitro* degradation (N=6 in each group)

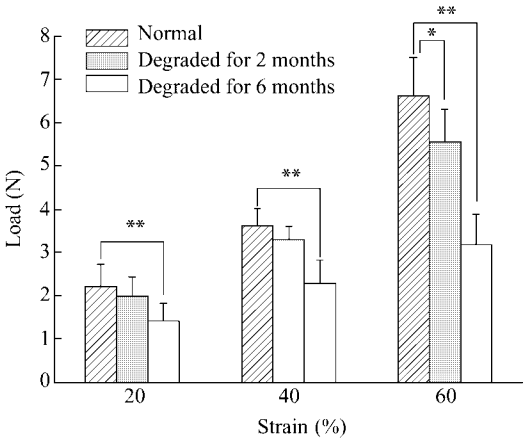

Fig. 4 Load-strain relationship during compressive loading (*$p < 0.05$, **$p < 0.01$, N=6 in each group)

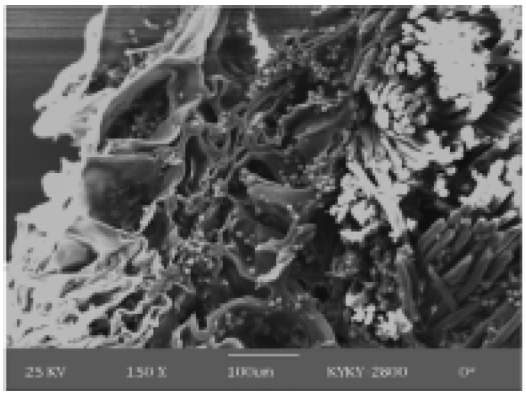

Fig. 5 Scanning electron micrograph of N2A cells on the cross section of the scaffold after co-cultured for 5 days (Scale bar=100 μm)

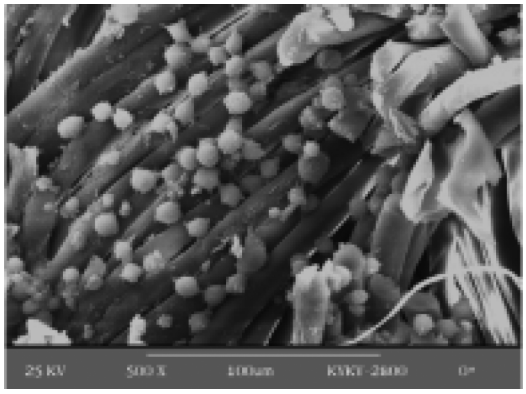

Fig. 6 Scanning electron micrograph of N2A cells on the fibrous inner layer of the tube after co-cultured for 5 days (Scale bar=100 μm)

3 Discussion

Tissue engineering has become a multidisciplinary subject with related technologies, such as polymer synthesis and fiber spinning, facilitating the design and development of novel scaffolds. Biotextiles, defined as structures composed of textile fibers and designed for use in a specific biological environment (e.g., surgical implants)[10], can meet all the criteria currently considered necessary for the design of ideal scaffold structures. These biotextile structures, whether woven, knitted, or nonwoven, are believed to be uniquely suited to serve as tissue engineering scaffolds and to compare favorably with other fabrication techniques[11]. Chitosan fibers and yarns are now available with the development of fiber science and chemical technologies. However, most of the research has emphasized their applications in fabricating hybrid woven materials and health care knitgoods. Only a few reports have discussed the possibility of using chitosan fibers in tissue engineering[12] with no known reports covering the application of chitosan yarns. This study is the first description of the fabrication of tubular scaffolds from chitosan yarns based on an industrial warp knitting process. Other studies are analyzing the use of chitosan fiber and yarns for other applications.

Compared with previous methods, the technique described here is more efficient, reproducible, and the resulting tubular scaffolds have a two-ply structure, which may be very favorable for soft-tissue engineering. The semipermeable outer layer not only forms a barrier to prevent in-growth of fibroblasts into the nerve gap when the scaffold is used as a artificial nerve graft, but also makes it possible for the transport of nutrients and metabolic wastes. The soft fibrous inner layer of the tube not only increases the overall surface area for cell attachment, but also enhances the tensile strength and suture ability by introducing bundles of chitsoan fibers.

The swelling measurements show that the scaffold outer diameter increased after hydration while the inner diameter remained essentially constant. The stable inner structure of the scaffolds after hydration or implantion into the body makes the scaffolds more suitable for artificial nerves, blood vessels, tracheas, etc. Both the mass and mechanical strength of the scaffolds decreased after the samples were degraded *in vitro*. In the first 2 months, the mass decreased a little more than in the next 4 months, whereas the maximum stress decreased less. The result indicates that some of the scaffold matrices undergo degradation more easily at the beginning, but these matrices are perhaps not

very important in maintaining the mechanical strength of the scaffold.

4 Conclusions

Chitosan fiber-based porous tubular scaffolds were produced using an industrial knitting process combined with a thermally induced phase separation technique. The resulting conduits have a biphasic wall structure, with a soft fibrous inner layer and a semipermeable outer layer. *In vitro* characterization shows that the scaffolds hold promise for regeneration of tissues requiring a tubular scaffold. These scaffolds are currently being further optimized to improve their capacity to facilitate regeneration of both peripheral nerves and blood vessel tissue.

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