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Development of fibrous biodegradable polymer conduits for guided nerve regeneration

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The technique of microbraiding with modification was employed as a novel method for the fabrication of fibrous tubular scaffolds for nerve tissue engineering purposes. The biodegradable polymers used in this study were poly(L-lactide-co-glycolide) (10:90) and chitosan. The polymeric fibers were microbraided around a Teflon mandrel to make it as a tubular construct. The conduits were then studied for their surface morphology, swelling behaviour and biocompatibility. The surface morphology was analysed by scanning electron microscope, swelling behaviour by weight increase due to water uptake and biocompatibility by *in vitro* cytotoxicity assessment in terms of cell morphology and cell viability by the MTT assay of polymer extract treated cells. These conduits may also be used for regeneration of tissues, which require tubular scaffolds such as blood vessel, spinal cord, intestine etc.

1. Introduction

When a nerve is cut or crushed and nerve function is lost, repair of the transected nerve is commonly aided by employing an autologous nerve graft, vein graft or arterial graft obtained from a second operative site from the patient and attached to the two ends of the severed nerve [1]. However, there are limitations inherent in this method, namely, limits to the length and thickness of the graft, the amount of the graft in supply and the degree of pain and neurological deficit suffered by the patient [2]. Thus, synthetic polymer tubes or conduits, provided that they are well tolerated *in vitro* show much promise as offering a promising alternative. The supply of the conduit is unlimited and the material can be fabricated to optimum dimensions for tissue regeneration. In addition, the consistency of material quality can be controlled, unlike natural materials (e.g. collagen), which may exhibit significant batch variation [3–11, 26].

Table I summarizes some of the recent work on nerve guide conduits. Tubes were fabricated using various polymers with porous but rigid structures. No attempt has been made in the fabrication of tubular conduits from fibers alone.

The present work deals with a new method of fabrication of fibrous tubular construct by a microbraiding technique. The fibrous conduits fabricated by the present method were highly porous and flexible, which is not observed in other solid porous tubular structures. The microbraided tubes were kink resistant and can be bent up to 180° and brought back to original shape without breakage, which is essential for tissue engineering purposes. The conduit was characterised for its swelling behaviour, microstructure and *in vitro* biocompatibility.

2. Materials and methods

2.1. Polymer

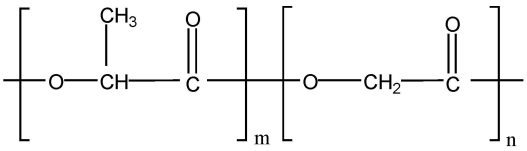
Biodegradable polymer fibers of 10:90 poly(L-lactide-co-glycolide) with molecular weight of 100,000 and 80% deacetylated chitosan from Tian Chuen Biomaterials Co. Ltd, Shanghai, China were used. The structural formulae of PLGA and chitosan are shown in Fig. 1 [12, 13].

2.2. Development and fabrication

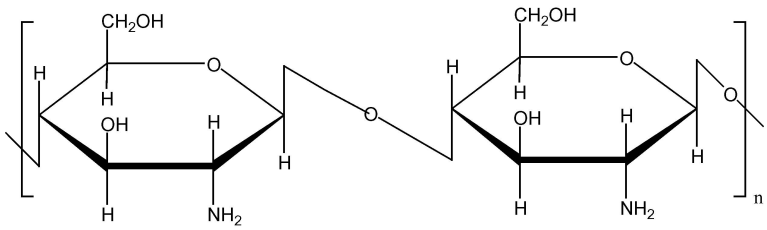
The method of microbraiding is normally used in the making of yarns from fiber and these yarns are used in the manufacture of fiber-reinforced composites for engineering applications. In this study, the conduits were developed by braiding the polymer fibers over a Teflon mandrel to obtain the desired construct. The required number of PLGA or chitosan fibers was wound onto the spindles of the microbraiding machine. Fig. 2 shows a schematic of the microbraiding process. The end of the fibers attached to the spindles was then pulled to the centre of the microbraiding machine. A continuous Teflon tube mandrel is inserted through the convergence point and forming point of the microbraiding machine from the bottom and pulled upwards. The machine is then switched ON to braid, and a tubular structure of infinite length is achieved. The diameter of the microbraided tubes can be controlled by controlling the diameter of the mandrel. The microbraided tube is then cut with

TABLE I Recent work on nerve guide conduits (The table summarizes some of the recent work on nerve guide conduits. In all of the research carried out so far, tubes were fabricated using various polymers with porous but rigid structures. No attempt has been made in the fabrication of tubular conduits from fibers alone. In the present study attempt has been done to fabricate fibrous conduits.)

Year	Conduit material and method of fabrication	Luminal filling	Gap length (mm)	Animal studied	Ref
2001	Poly(phosphoester) Dip-coating	None	10	Rat	[14]
2001	Poly(phosphoester) Dip-coating	None	10	Rat	[20]
2001	Poly(DLLA-ε-CL) Dip-coating	Modified denatured muscle	15	Rat	[7]
2001	Collagen filaments	Collagen filaments	20	Rat	[43]
2000	PLGA/Laminin Foam-processing technique employing low-pressure injection molding.	Schwann cells	7	Rat	[36]
2000	PLLA Combined solvent casting, extrusion and particulate leaching technique.	None	12	Rat	[38]
2000	PGA mesh, collagen Dipcoating	Laminin-coated collagen filaments	80	Dog	[45]
1999	Nerve graft	VEGF/NGF	10	Rat	[42]
1998	Collagen, Silicon Commercially available. Collagen tubes from Integra Life sciences, Inc., Plainsboro, NJ. Silicon tubes from Silastic Medical Grade Tubing, Dow-Corning Co., Midland, MI.	Collagen-GAG matrix	10	Rat	[34]
1998	PLLA/PLGA Dip-molding technique	Schwann cells	20	Rat	[35]
1998	Biodegradable glass Commercially available	None	Constriction	Sheep	[39]
1997	PLA/CL Dip-coating technique	None	10	Rat	[8]
1997	Vein	Acellular muscle grafts	20	Rat	[37]
1997	Silicone Commercially available tube	Polyamide filaments	15	Rat	[41]
1996	PLLA/CL Dip-coating	None	10	Rat	[15]
1996	PLA/CL Dip-coating	None	10	Rat	[11]
1996	Silicone	Collagen/ prosaposin	6	Guinea	[21]
1996	PLLA/CL Dip-coating technique	Modified denatured muscle	15	Rat	[22]
1995	Polyethylene Commercially available nerve guide, Intramedic, Fisher Science, Springfield, NJ.	Hyaluronic acid	10	Rat	[23]
1995	Polyethylene Commercially available nerve guide, Intramedic, Fisher Science, Springfield, NJ.	NTF, GF	10	Rat	[24]
1995	Vein segment, PLLA/CL Dip-coating	None	10	Rat	[9]
1994	Polyethylene Commercially available	None	4	Rat	[25]
1994	Collagen Type IV Mold set	α-MSH, b-FGF	7	Rat	[26]
1993	PLLA/PCL Dip-coating	None	10	Rat	[27]
1993	Vein segment	Muscle	10–20	Rat	[28]
1991	Collagen Commercially available conduit from Colla-Tec, Inc., Plainsboro, N.J.	None	4	Monkey	[29]
1991	HEB (hydrophilic elastomeric biopolymer), PGA. Commercially available from Aquavene biopolymer, CA and Davis and Geck Inc, NY.	None	5	Rat	[30]
1989	PTFE Commercially available	None	4	Mice	[40]
1989	Silicon	None	Case history		[31]
1987	PVC/acrylic Commercially available, Amicon Corp., Lexington, Massachusetts	Collagen, laminin	4	Mice	[32]
1987	PVDF	None	4	Mice	[33]



Poly(L-lactide-co-glycolide)



Chitosan

Figure 1 Structural formulae of poly(L-lactide-co-glycolide) and chitosan.

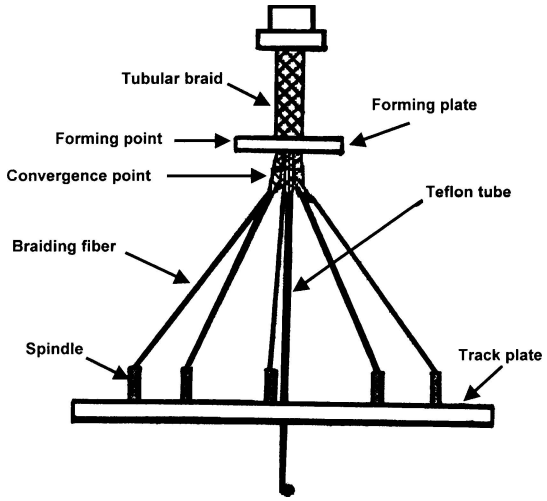


Figure 2 Schematic of microbraiding process.

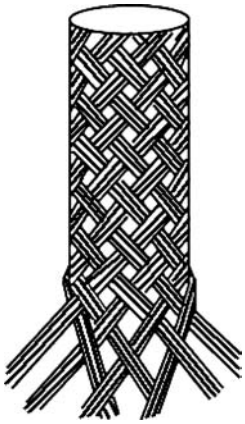


Figure 3 Schematic of a microbraided tubular construct.

a heated penknife to melt the ends of the fibers, thus keeping them intact and preventing the edges from unravelling. Chitosan fibers do not melt on heating. Thus the chitosan microbraided tube was cut with ordinary scissors and the ends dipped in actetic acid solvent to keep the fibers at the two ends of the tube intact and prevent from unravelling. After the microbraided tubes were cut into the required length, the Teflon mandrel was removed leaving the fibrous conduit. Fig. 3 shows the schematic of a microbraided conduit. The porosity of the tubular structure obtained by microbraiding can be varied by changing any of the following parameters, the braiding angle, the number of fibers in a spindle, the number of monofilaments in a fiber and the number of spindles.

PLGA and chitosan fibers have the attractive properties of biocompatibility and biodegradability and were used in the present investigation. The fibrous conduits fabricated by microbraiding have the advantage of high flexibility, easy suturability, permeability and variable wall thickness.

2.3. Microstructural characterization

SEM analysis of the tubular conduits were carried out at three different magnifications to study the surface morphology, cross linking of fibers and porosity of the conduits using a JEOL JSM-5800LV scanning electron microscope at an accelerating voltage of 15 kV. The braiding angle measurement was also carried out on the microstructure.

2.4. Swelling test

Swelling tests of the tubular conduits were performed by measuring the weight change in PBS due to water absorption. The conduits were sterilized and their initial mass measured. Then it was allowed to swell by placing in phosphate buffer pH 7.4, sealed and incubated at 37 °C. At selected time points, the conduits were removed from the solution, blotted with an absorbent tissue and weighed for weight increase [14]. In total, twelve samples were studied. The percentage weight remaining ($W\%$) was calculated according to the following equation

$$W(\%) = (W_f / W_i) \times 100$$

where, W_f is the weight of swollen conduit and W_i is the initial weight of the conduit.

2.5. Biocompatibility assessment

In vitro biocompatibility assessment was carried out to assess the cytotoxicity of different types of cells in terms of the cell morphology and cell viability by MTT assay.

2.5.1. Cell morphology

In order to characterize the morphology of the cells attached to the conduits, 80 mg of PLGA and Chitosan fibers were sterilized in 70% sterilized ethanol, dried and incubated in 5 ml of DMEM (Dulbecco's Modified Eagle's Medium) for 72 h at 37 °C for extraction. The extract was then collected by centrifuging the polymer contents and obtaining the supernatant. Four types of cells, primary neurons, primary fibroblasts, undifferentiated rat pheochromocytoma PC12 and C17.2 nerve stem cells were used in this study. The cultured cells were plated into 24-well plate at a density of 1×10^4 cells/well and kept in an incubator for 2–4 h. 0.5 ml of the extract was then added to the plated wells. Control wells were prepared by adding 0.5 ml of DMEM to the plated wells. The cells were cultured with the extract for 3 days. It was then morphologically characterized.

2.5.2. MTT assay

In order to carry out the MTT Assay, PC12 cells were used. 80 mg of PLGA or Chitosan fibers were sterilized in sterilized ethanol and incubated in 5 ml of culture medium for 72 h at 37 °C. The suspension was then centrifuged and the supernatant was collected. Serial dilutions of 1, 2, 4, 8 mg/ml of the extract were made using growth medium. PC12 cultured cells were re-suspended in the culture medium and plated into 96-well plate at a density of 1×10^4 cells/well. The plates were incubated for 4 h at 37 °C. The medium was then replaced by the extract dilutions. Control plates were prepared by using culture medium. They were then cultured for 3 days at 37 °C. After incubation the cell culture was treated with 20 μl/well of 5 mg/ml MTT. It was further incubated for 4 h at 37 °C. The supernatant was discarded and the formazan crystals were solubilized by adding 100 μl of DMSO solution. The plates were kept in room temperature for 2 h and stabilized. The optical density of each well was read at 595 nm using a BIO-RAD Model 550 Microplate Reader.

3. Results and discussion

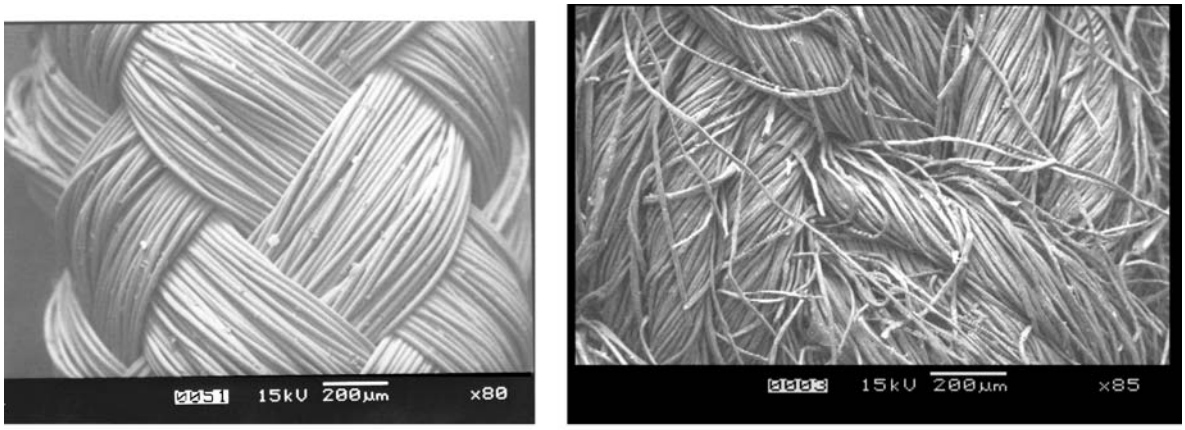
3.1. Developed conduit

In the present work, conduits were fabricated from fibers alone providing many advantages over the other solid polymeric tubes studied or reported in the literature. Two different conduits from PLGA and chitosan fibers were fabricated by microbraiding technique. Fig. 4 shows the macrograph of the conduits. The developed conduits have a high enough flexibility necessary to adapt well inside a living system. The fibrous structure of the conduit makes it easy to be sutured to the tissues inside a living body. Also the conduit is strong enough to remain without tearing at the sutured site. The highly porous structure of the conduit makes it highly permeable, which in the case of nerve regeneration is essential for the entry of nutrients into the conduit lumen to promote nerve regeneration and at the same time has the necessary barrier to prevent the infiltration of unwanted tissues into the conduit from outside. The developed conduits also do not easily suffer tube breakage, which is often encountered with other types of solid polymer conduit. It is easy to fabricate the tubular conduit by the present microbraiding technique into any required length and diameter and has no dimensional limitations in fabrication. The present method is suitable to fabricate a conduit or scaffold with any required biodegradable material available as fiber. This method of fabrication does not involve heating or chemical reactions during tubulation. Thus a material, which is not thermally or chemically stable can only be tubulated by the microbraiding technique provided it is in fiber form.

3.2. Microstructural characterisation

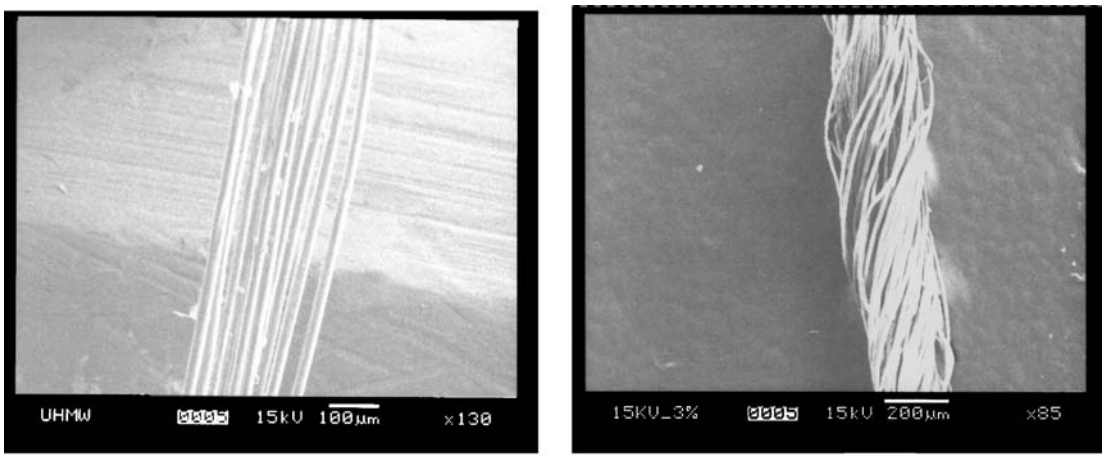
The microstructure of the PLGA and chitosan conduits were determined by scanning electron microscopy at different magnifications of 80×, 170× and 300×, to determine the surface morphology, pore size and braiding angle. Fig. 5 shows the micrograph of the tubular microbraided conduits at 80× or 85×. The surface morphology as seen in the micrograph is a porous structure with cross-linked fibers. The PLGA fibers were multifilament type with no twisting, whereas the chitosan fibers were multifilament and twisted, as seen in Fig. 6. The pore size of the pores found in between the cross linking of the fibers was measured to be 50–100 μm, in the case of PLGA scaffold, Fig. 7a(A). The fibers in the chitosan conduit were twisted and also showed a

Figure 4 Macrograph of the tubular scaffold (A) PLGA (B) Chitosan.



(A) (B)

Figure 5 SEM micrograph of the tubular scaffolds (A) PLGA (B) Chitosan.



(A) (B)

Figure 6 Scanning electron micrograph of (A) PLGA fiber, multifilament and straight (B) Chitosan fiber, multifilament and twisted.

threading behaviour. Thus the pores were blocked and not observed, Fig. 7a(B). The braiding angle was set at 45° for both PLGA and Chitosan scaffold as shown in Fig. 7(b).

3.3. Swelling test

Swelling of the conduits or scaffolds is a common feature seen in biodegradable tubular constructs and it is commonly due to water uptake into the porous structure. Also, as the degradation of the conduit proceeds, the polymer is broken down into smaller degradation products, which may absorb water and enhance swelling. In this study, PLGA conduits demonstrated no increase in weight and hence no sample swelling, see Fig. 8(a). This is advantageous for nerve tissue engineering purposes, as the lumen space will be kept constant. In contrast, the chitosan conduits showed almost a 60% weight increase and hence significant polymer swelling, see Fig. 8(b). The swelling caused a decrease in lumen space of the tubular conduit. Hence in the case of chitosan scaffolds, it is necessary to take into consideration the swelling behaviour before designing the conduit for a particular purpose [15, 16]. The problem can be solved by increasing the internal diameter of the tube to give room for swelling of the conduit. Thus, type of material used in biodegradable tubular scaffold determines the degree of conduit swelling.

3.4. Biocompatibility assessment

3.4.1. Cell morphology

The cell morphology is an important factor in determining the biocompatibility of a biomaterial. The extracts made from PLGA polymer did not alter the cell morphology compared to controls in any of the four cell types tested after treatment for 3 days. The tested cell types include primary neurons, primary fibroblasts, PC12 cells and C17.2 nerve stem cells. Fig. 9 shows the cell morphology of the different cell types in both extract treated and control plates for the PLGA polymer. There was no influence of the polymer extract on the morphology of the cells, which suggested good biocompatibility of PLGA material.

For extracts made from chitosan polymer, the cell morphology was also unaffected for three of the four cell types studied, primary neurons, primary fibroblasts and PC12 cells. However, for C17.2 nerve stem cells, cell death was shown to occur after 3 days of culture, Fig. 10. The morphology of control plates is shown in Fig. 9. Thus only PLGA showed good biocompatibility to all the four cell types tested.

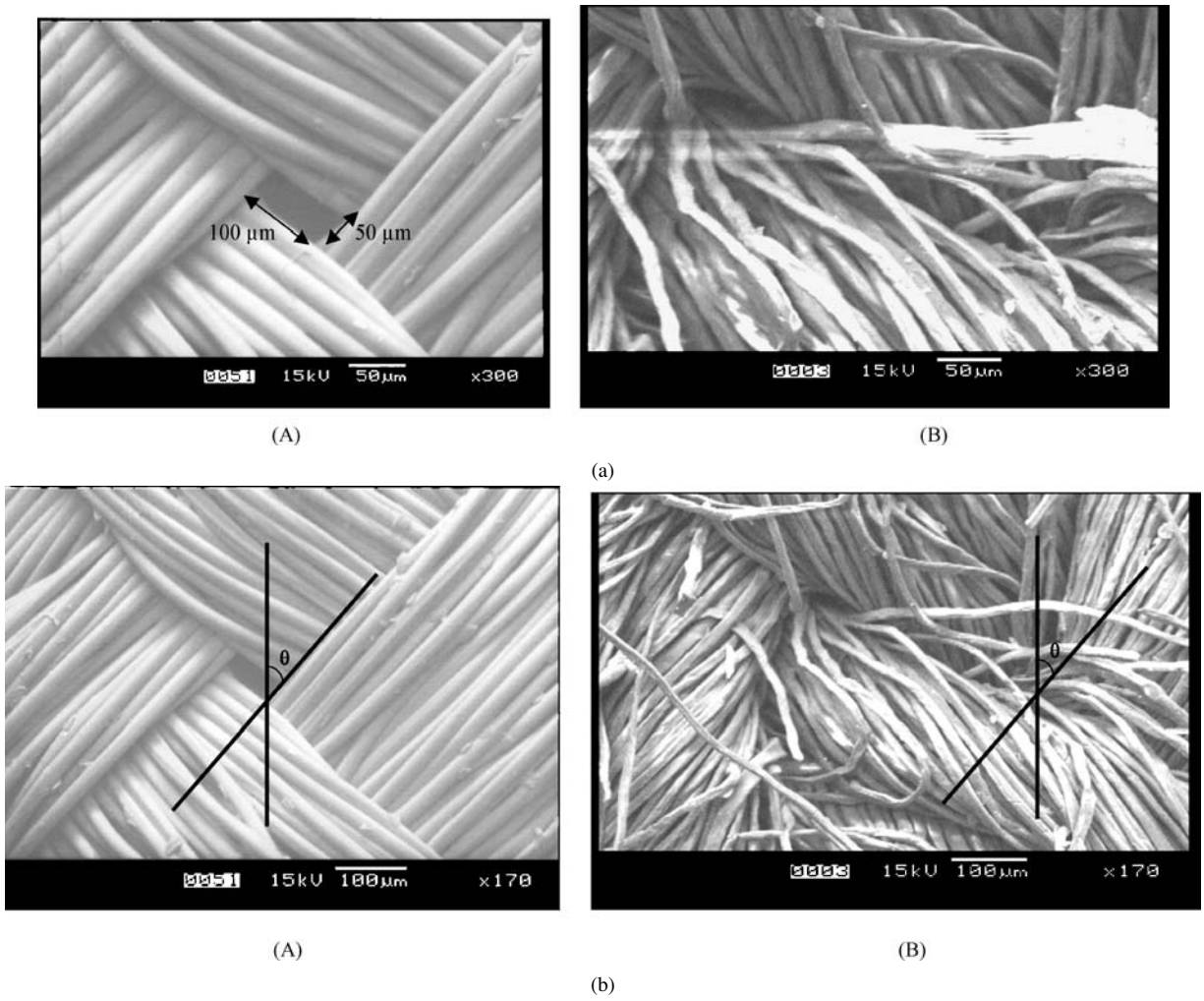


Figure 7 (a) Scanning electron micrograph showing the pore size of (A) PLGA scaffold (B) Chitosan scaffold. (b) Scanning electron micrograph showing the braiding angle θ of (A) PLGA scaffold (B) Chitosan scaffold.

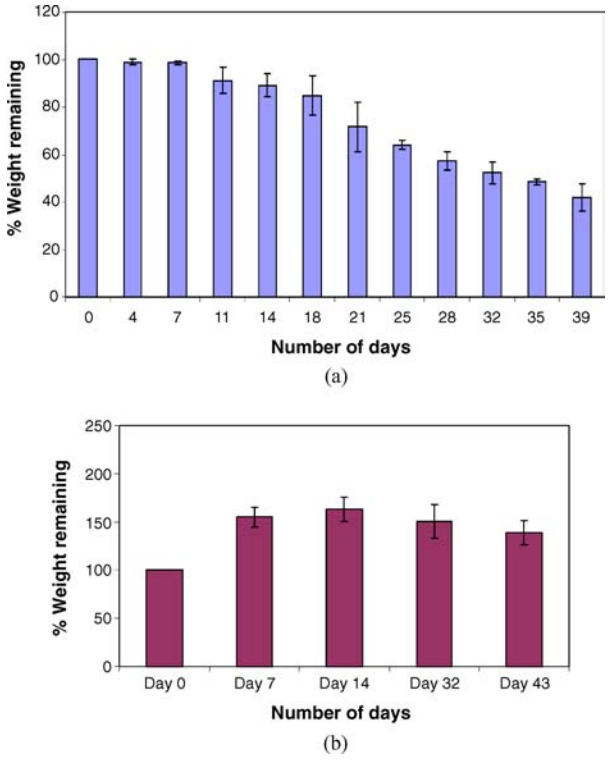


Figure 8 (a) *In vitro* swelling test result of (a) PLGA scaffold and (b) Chitosan scaffold.

3.4.2. MTT assay

MTT reagent is a pale yellow substrate, which produces a dark blue formazan product when incubated with viable cells. Therefore, the level of the reduction of MTT into formazan can reflect the level of cell metabolism [17, 18]. The MTT results are presented in Fig. 11. It was observed that in spite of the decrease in the absorbance with an increase in the extract concentration, both the materials did not show complete cytotoxicity for PC12 cells. The percentage absorbance was found to be greater than 50% even for dilutions upto 8 mg/ml. These results are typical for biodegradable polymers, as their degradation tends to induce a certain extent of cytotoxicity, especially when they induce a strong pH drop [19]. Hence PLGA, which degrades faster, shows a slightly higher degree of cytotoxicity than chitosan. The extracts of both PLGA and Chitosan however did not induce deleterious effects on PC12 cells as seen through the MTT assay, indicating better tolerance of the cells and also absence of cytotoxicity for material leach out. The potential of the PLGA conduit for nerve regeneration was carried out on the right sciatic nerve of the rat. It was successful and is described in detail elsewhere [44]. The fibrous conduits showed no tube breakage and showed successful nerve regeneration.

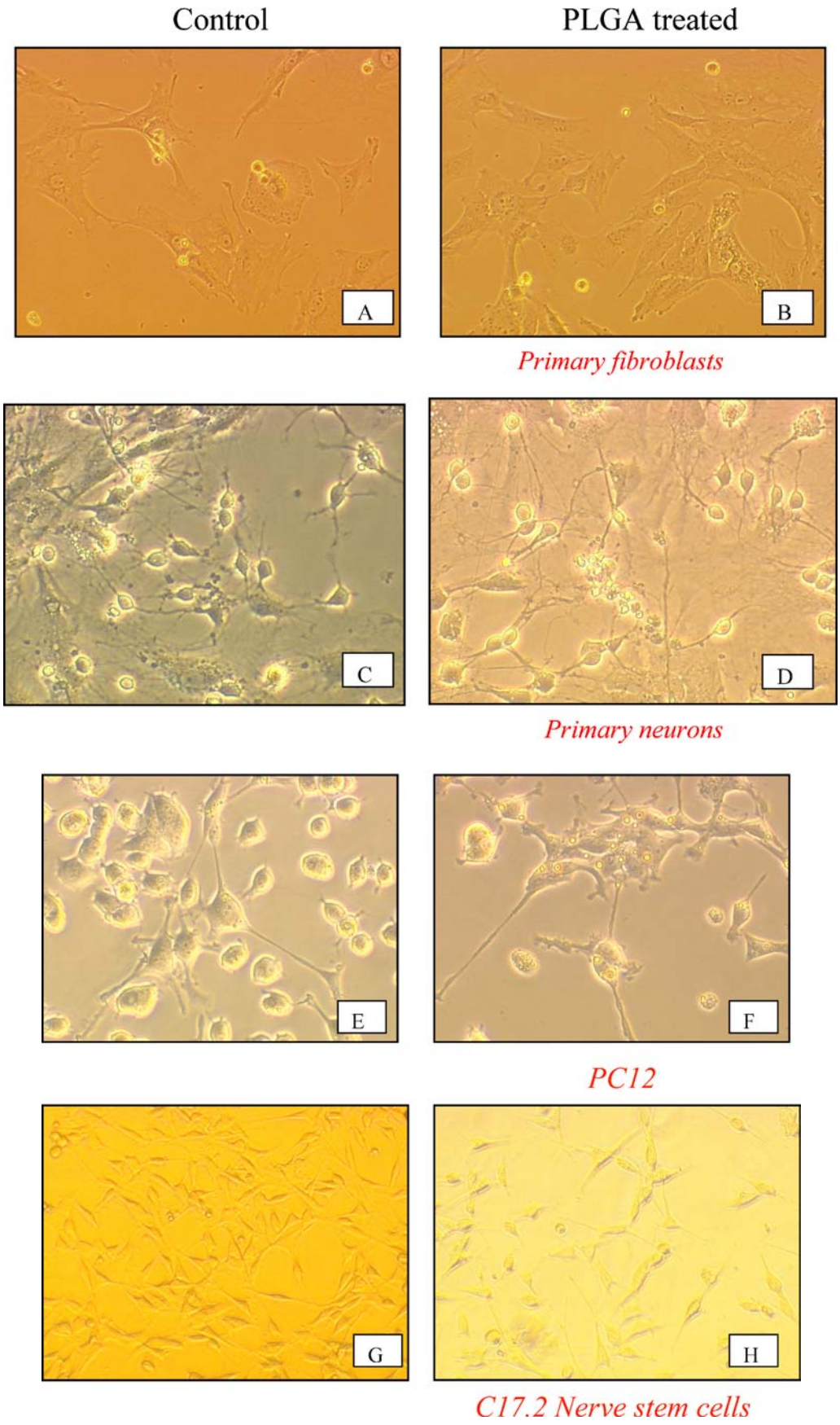


Figure 9 The biocompatibility of PLGA material. The cell morphology of rat primary fibroblasts, primary neurons, PC12 and C17.2 cells in control (A), (C), (E), (G) and in PLGA treated (B), (D), (F), (H) cell cultures—magnification 200×. The cell morphologies are not affected after treatment with extracts from PLGA.

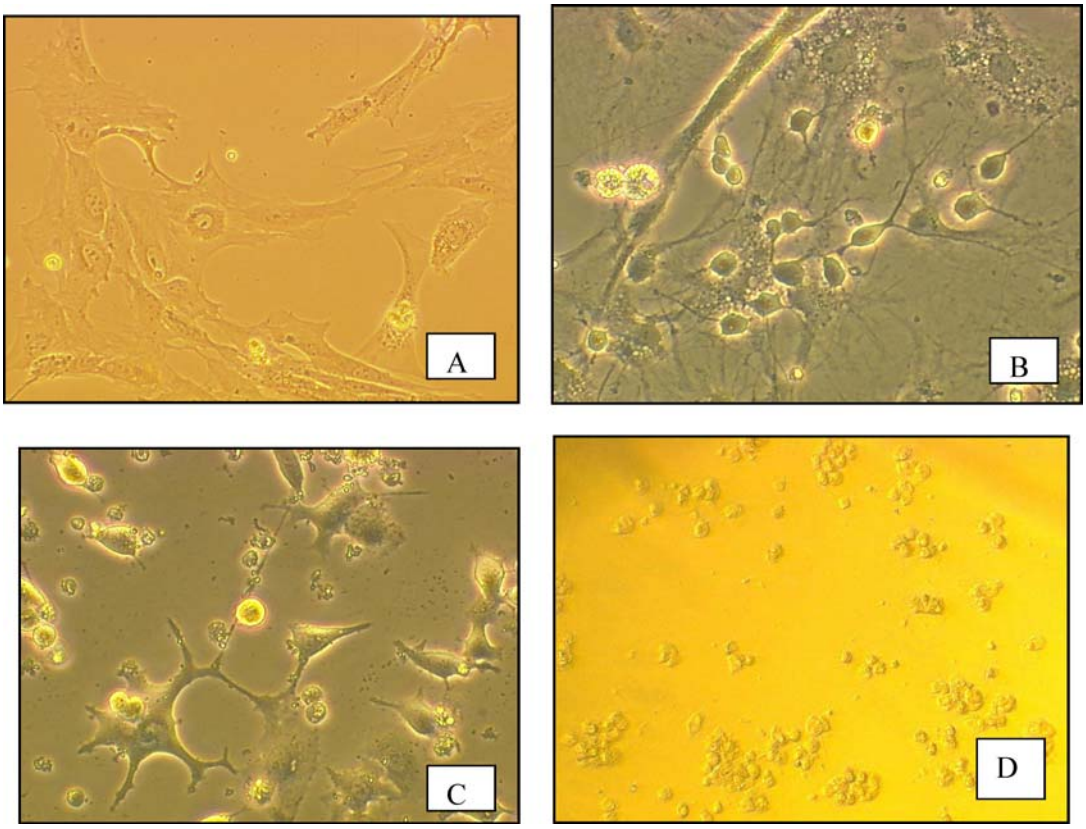


Figure 10 The biocompatibility of Chitosan material. The cell morphology of rat primary fibroblasts, primary neurons, PC12 and C17.2 cells (A), (B), (C), (D)—magnification 200×, (A–C) are not affected after treatment with extracts from Chitosan, (D) C17.2 showed cell death on treatment with extracts from chitosan.

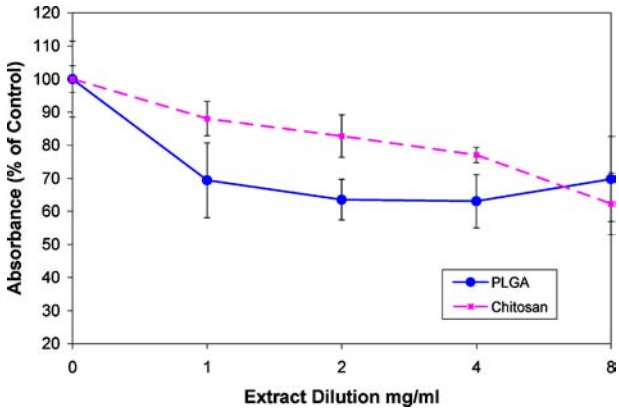


Figure 11 MTT assay. Formazan absorbance (% of control) after 72 h of PC12 cell growth with extracts of PLGA and Chitosan.

4. Conclusions

In conclusion, a method to fabricate fibrous, porous, flexible, biodegradable and strong tubular scaffold was developed. The fabrication method was successfully applied to two different polymer fibers, PLGA and chitosan. The pore morphology of these tubular scaffolds was mainly due to the cross alignment of fibers. The pore size can be varied by changing the braiding angle, the number of fibers in the spindle, the number of monofilaments in the fiber or the number of spindles used in the microbraiding machine. PLGA conduit exhibited negligible or no swelling thus maintaining the dimensional integrity. However, the chitosan conduit showed a maximum of 60% swelling, which has to be taken into consideration before designing the scaffold for practical applications. Both PLGA and Chitosan scaffolds showed good biocompatibility. Cell morphology was not altered and was similar in both control and polymer extract treated well plates except for chitosan treated C17.2 nerve stem cells. MTT assay showed good viability of cells necessary for the materials to be biocompatible. Taking into account the swelling of chitosan conduit and death of C17.2 nerve stem cells in the biocompatibility assessment of chitosan, a conduit of PLGA polymer is much preferred than chitosan. Both scaffolds are expected to be suitable for other tissue engineering purposes as such or with seeded cells or impregnated with microspheres loaded with tissue-inductive factors.

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