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Poly(l-lactide-co-glycolide) biodegradable microfibers and electrospun nanofibers for nerve tissue engineering: an in vitro study

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Abstract For tissue engineering applications, the distribution and growth of cells on a scaffold are key requirements. The potential of biodegradable poly(l-lactide-co-glycolide) (PLGA) polymer with different microstructures, as scaffolds for nerve tissue engineering was investigated. In this study, an attempt was made to develop porous nanofibrous scaffolds by the electrospinning method. In this process, polymer fibers with diameters in the nanometer range are formed by subjecting a polymer fluid jet to a high electric field. Attempt was also made to develop microbraided and aligned microfiber scaffolds. A polymer film scaffold was made by solvent casting method. C17.2 nerve stem cells were seeded and cultured on all the four different types of scaffolds under static conditions for 3 days. Scanning electron micrographs showed that the nerve stem cells adhered and differentiated on all the scaffolds and supported neurite outgrowth. Interesting observation was seen in the aligned microfiber scaffolds, where the C17.2 nerve stem cells attached and differentiated along the direction of the fibers. The size and shape of the cell-polymer constructs remained intact. The present study suggests that PLGA is a potential scaffold for nerve tissue engineering and predicts the orientation and growth of nerve stem cells on the scaffold.

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Introduction

Traumatic injuries to the adult mammalian central nervous system (CNS) cause severe and irreparable disabilities. Even limited local injuries to the brain or the spinal cord have devastating consequences due to the inability of the CNS to regenerate injured nerve fibers or to react with structural plasticity [1]. Thus only a few studies are currently available in literature on nerve tissue engineering, in particular the CNS [1, 2]. Recent discoveries have however challenged this dogma. In particular, a more complete understanding of developmental neurobiology has provided an insight into possible ways in which neuronal regeneration in the CNS may be encouraged. Knowledge of the role of neurotrophic factors has provided one set of strategies, which may be useful in enhancing CNS regeneration. These factors can now even be delivered to injury sites by transplantation of genetically modified cells. Another strategy showing great promise is the discovery and isolation of neural stem cells from adult CNS tissue. It may become possible to grow such cells in the laboratory and use these to replace injured or dead neurons [3]. In this context polymers can be used as scaffold to promote cell adhesion, maintenance of differentiated cell function without hindering proliferation to act as a template to organize and direct the growth of cells and help in the function of extracellular matrix. The scaffolds as cell and tissue carriers can be fabricated using either natural or synthetic polymers [4].

The present study explores the potential of using poly(l-lactide-co-glycolide) (PLGA) biodegradable polymer as scaffolds for nerve tissue engineering, acting as carriers for cells. Scaffolds were made using PLGA microfiber and PLGA nanofiber prepared by

the electrospining process. Electrospinning is a process for obtaining fibers in the nanometer scale and is an area receiving attention in nanotechnology [5–8]. In vitro studies were carried out using C-17.2 nerve stem cells. C17.2 is a multipotent neural stem cell line generated via retrovirus-mediated *v*-myc transfer into murine cerebellar progenitor cells. These cells can be used as neuron precursors since they are involved in the normal development of cerebellum, embryonic neocortex and other structures upon implantation into mouse germinal zones [9–11].

Materials and methods

Materials

Poly(l-lactide-co-glycolide) (PLGA 10:90) microfibers with molecular weight of 100,000 purchased from Tian Chuen Biomaterials Co. Ltd, Shanghai, China were used in the present study. The solvent, hexafluroisopropanol (HFIP) was supplied by Sigma, USA and used as received. For cell culture study, phosphate buffer saline (PBS) of pH 7.4, Dulbecco's Modified Eagle Medium (DMEM) were obtained from Sigma, USA and DMEM/Nutrient Mixture F-12 Ham (DMEM/F-12 1:1 mixture) and N-2 supplement were obtained from Gibco, USA. Glutaraldehyde and Osmium tetroxide (Sigma) were used to post-fix the cells before SEM observation.

Fabrication of PLGA nanofibrous scaffolds by electrospinning

The PLGA nanofibrous scaffolds were fabricated by electrospinning process. HFIP was used as a solvent to dissolve PLGA microfibers under gentle stirring to obtain a 7 wt% solution. Fig. 1 shows a schematic picture of an electrospinning setup. The polymer solution was placed in a 5 mL plastic syringe fitted to a needle with a tip diameter of 0.4 mm. A high voltage of 12 kV was applied to the needle using a high voltage power supply (Gamma High Voltage Research). The ground collection plate of aluminum foil was located at a fixed distance of 10 cm from the needle tip. A syringe pump was used to feed the polymer solution to the needle tip at a feeding rate of 1 mL/h. The polymer solution formed a Taylor cone at the tip of the needle by the combined force of gravity and electrostatic charge. A positively charged jet was formed from the Taylor cone and was sprayed to the grounded aluminum foil target. The nanofibers were collected over the coverslips placed on the aluminum foil. The as spun

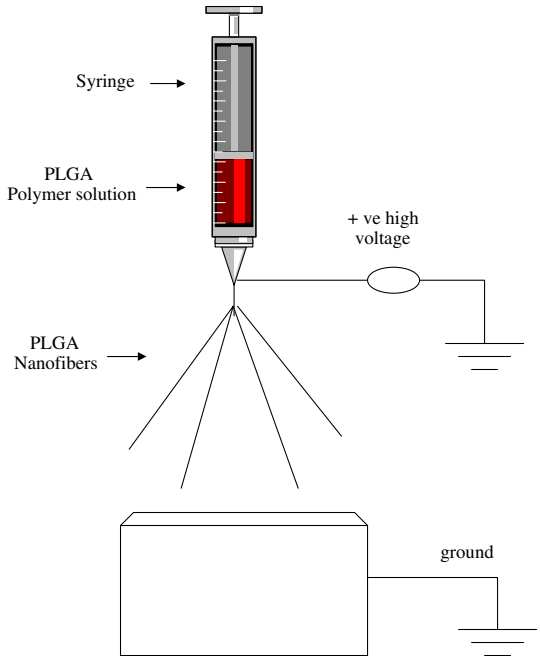

Fig. 1 Shows a schematic picture of an electrospinning setup

nanofibers were dried under vacuum at room temperature over night. For the characterization, electrospun nanofibers were sputter coated with gold using a JEOL JFC-1200 Fine Coater. The morphology of the electrospun nanofibers was observed using JEOL JSM-5800LV scanning electron microscope at an accelerating voltage of 5 kV. The electrospun nanofiber scaffolds were sterilized in an autoclave at 120 °C for 20 min before cell seeding.

Fabrication of PLGA microfiber scaffolds by microbraiding

The PLGA microfiber scaffolds were fabricated by microbraiding technique. The required number of PLGA fibers was wound on the spindles of the microbraiding machine. The ends of the fiber from the spindles were then pulled to the center of the microbraiding machine. A Teflon mandrel was 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 obtained. Fig. 2 shows a schematic picture of a microbraiding machine. The tube is then cut to the required length with a heated penknife to prevent the fibers from unwinding and the mandrel removed. The tubular structure was cut open to make it as a flat structure for cell culture. The microbraided scaffold was sterilized with 70% ethanol for 1 h and washed with PBS prior to cell seeding.

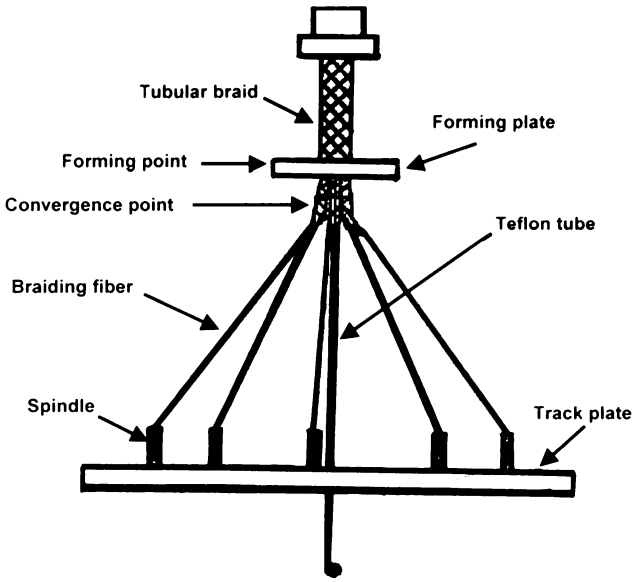

Fig. 2 Schematic picture of a microbraiding process

Fabrication of aligned PLGA microfiber scaffold

Aligned microfiber scaffolds were fabricated by carefully winding the microfibers manually on a glass slip to obtain a linear arrangement of the fibers. It was wound tightly to minimize the gap between the fibers. The scaffold was sterilized with 70% ethanol for 1 h and washed with PBS prior to cell seeding.

Fabrication of PLGA film by solvent casting

Thin PLGA films were fabricated by the solvent casting method. The PLGA microfibers were dissolved in HFIP solvent with stirring to obtain a 7 wt% solution. Approximately 1 mL of the solution was then cast onto the bottom of a 4 cm diameter glass petridish so as to obtain a thin layer of the solution. The solvent was allowed to evaporate and the petridish was placed in vacuum overnight to remove any remaining solvent. The film was then peeled off from the dish and cut to 13 mm diameter and placed over glass coverslip. The scaffold was sterilized with 70% ethanol for 1 hr and washed with PBS prior to cell seeding.

In vitro cell culture studies

C17.2 stem cells were cultured over the four different PLGA scaffolds prepared—electrospun nanofiber scaffold, microbraided microfiber scaffold, aligned microfiber scaffold and polymer film.

C17.2 cells were maintained in DMEM culture medium supplemented with 10% fetal calf serum, 5% horse serum and 1% penicillin–streptomycin as well. The cells were split into 1:2 every two days. Before seeding C17.2 cells onto the scaffolds, cells were detached from the cell culture flask and viable cells were counted by trypan blue assay. The cells were then seeded onto the scaffolds inside a 24-well plate with a density of 5×10^4 per well in the culture medium of DMEM/F12 containing N-2 supplement. One of the wells without the differentiating medium N-2 was used as a reference. Once the media was added, the culture wells were covered with a sterile cover and placed in an incubator for 3 days. After 3 days of culture in the incubator, the medium was discarded and the individual scaffolds were taken for SEM observation.

Scanning electron microscopic analysis

After 3 days of culture, SEM micrographs were taken when C17.2 cells were attached on the different scaffolds. The cellular constructs were harvested, rinsed with PBS twice and then fixed with 2.5% glutaraldehyde for about 10–20 min and post fixed with 1% OsO_4 for 20 min. The samples were then gently rinsed with PBS and dehydrated through a series of increasing concentration of ethanol solutions, 50% ethanol for 10 min; 70% ethanol for 10 min; 90% ethanol for 10 min and 100% ethanol for 10 min (twice). The samples were then air-dried in a fume hood overnight. The dry cellular scaffolds were mounted on to a stub, sputter coated with gold and observed under the SEM.

Results and discussion

In vitro cell culture study

Neuron cells are commonly cultured by coating natural polymers (laminin, peptides, collagen) and synthetic polymers (PLL and Matrigel) onto culture plates [12–18]. Most of the neuron cell cultures in literature have not made use of any scaffold and there is no report that biodegradable polymers could be used as synthetic substrates to support neuron differentiation. In the present study, PLGA biodegradable polymer scaffolds were investigated for C17.2 cell culture to support neuron differentiation that could be used for nerve tissue engineering purposes in the CNS. SEM examination demonstrated the attachment of the cells on the scaffolds by 3 days of culture. The SEM micrographs showed a random distribution of the cells on the polymer scaffolds.

Electrospinning is capable of producing fibers in the nanometer diameter range that are typically deposited in the form of non-woven fabrics. Electrospinning is driven by electrostatic forces and requires only small amounts of polymer. The latter feature is particularly useful for processing newly synthesized polymers that are not available in large quantities. Due to the small fiber diameters and the overall porous structure, electrospun fabrics have a high specific surface area that is beneficial for tissue engineering applications. Also, the overall topographical resemblance of electrospun fabrics to extracellular matrices makes it suitable for tissue engineering purposes [5–8].

In the present study it was observed that C17.2 NSC's were randomly distributed on the PLGA electrospun nanofiber scaffolds Fig. 3.1 and the differentiated neurons had their neurites oriented in random directions Fig. 3.2(a). SEM pictures showed the neurite formation in the stem cells indicating differentiation has occurred. Most of the neurons were found on the surface of the nanofiber scaffold. The neurite length was longer than two times the cell body, Fig. 3.2(b). The distribution of undifferentiated cells, the reference, on the PLGA nanofiber scaffold is seen in Fig. 3.2(c). A study by Yoshimoto et al. using mesenchymal stem cells cultured on electrospun PCL scaffold reported similar random distribution of the cells on the nanofiber scaffold [6].

In the case of PLGA microbraided scaffolds Fig. 4.1, the cells were found to align along the direction of the fiber orientation. It was interesting to note that the neurites differentiated along the fiber direction Fig. 4.2(a) and (b). The number of neurons on the surface of the microbraided scaffold was less compared to the nanofiber scaffold. The distribution of undifferentiated cells on the microbraided scaffold is shown in Fig. 4.2(c). The number of neurons on the surface of the microbraided scaffold was less compared to the nanofiber scaffold because of the smaller diameter of nanofiber scaffolds.

In the PLGA aligned microfiber scaffold Fig. 5.1, the cells were found oriented along the direction of the fiber Fig. 5.2(a) and (b). The neurite of the differentiated neuron extended along the fiber direction. This was an interesting observation as there is very limited study reported to bring about alignment of cells in a particular direction. In 1995 Stothard reported that fibroblasts aligned to the long axis of the grooves with organization of actin and other cytoskeleton elements in an orientation parallel to the grooves [19]. The aligned microfiber cell-scaffolds can be used for tissue engineering purpose in the CNS. The reference

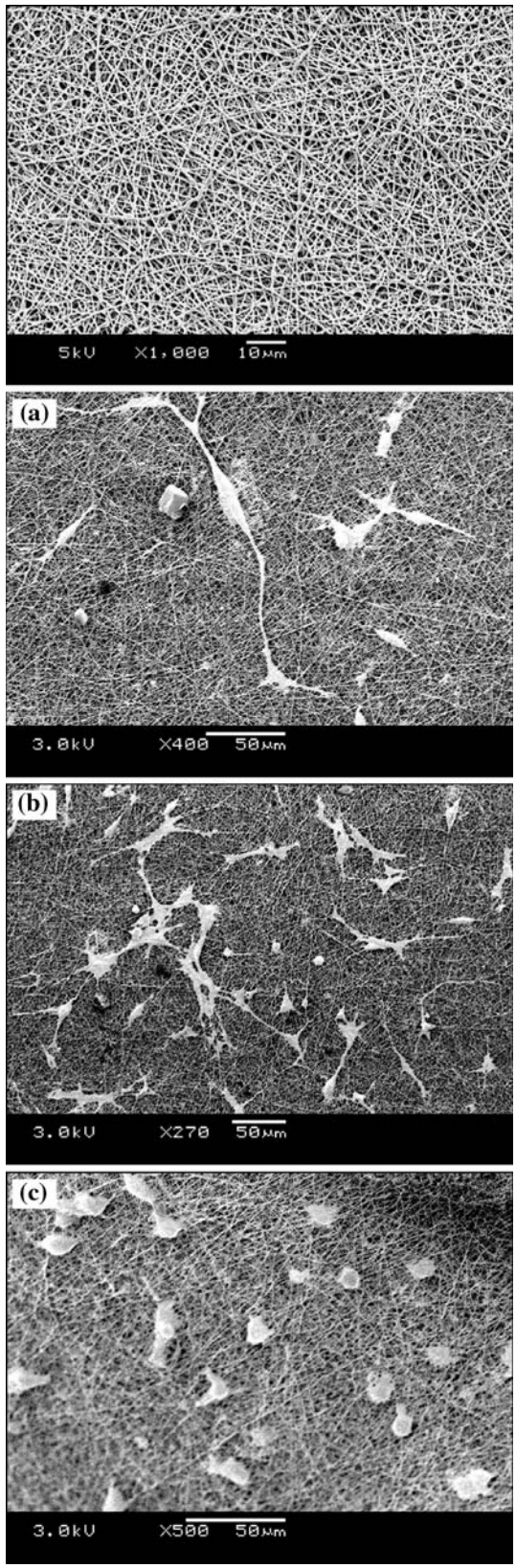

Fig. 3 1: An electrospun PLGA nanofiber scaffold. 2(**a**) and (**b**) shows the differentiated C17.2 cells attached on nanofiber scaffold, (**c**) shows the reference undifferentiated C17.2 cells

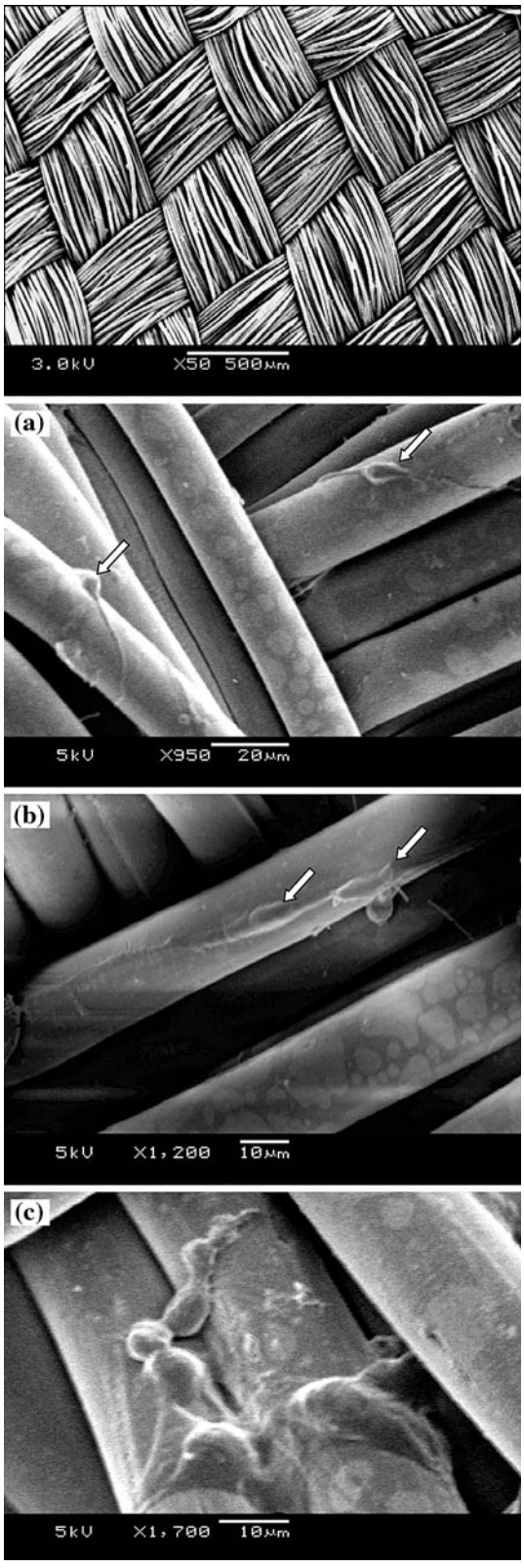

Fig. 4 1: A microbraided PLGA microfiber scaffold. 2(**a**) and (**b**) Shows C17.2 cells attached on the microbraided scaffold. The cells are seen aligned along the direction of fiber orientation, (**c**) shows the distribution of the reference undifferentiated cells

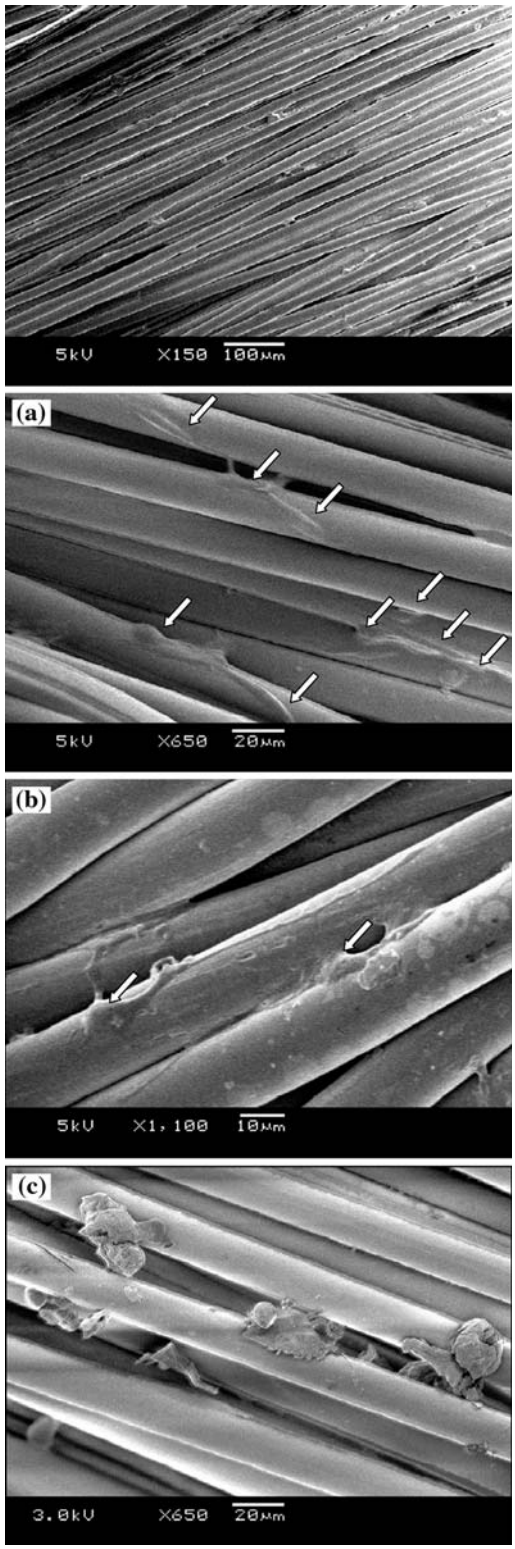

Fig. 5 1: Shows aligned PLGA microfiber scaffold. 2(**a**) and (**b**) Shows C17.2 cells attached on the aligned microfiber scaffold. The cells are found to attach on the fiber and is aligned along the direction of the fiber. The differentiated neurite is seen to grow along the direction of the fiber (**c**) shows the reference undifferentiated cells

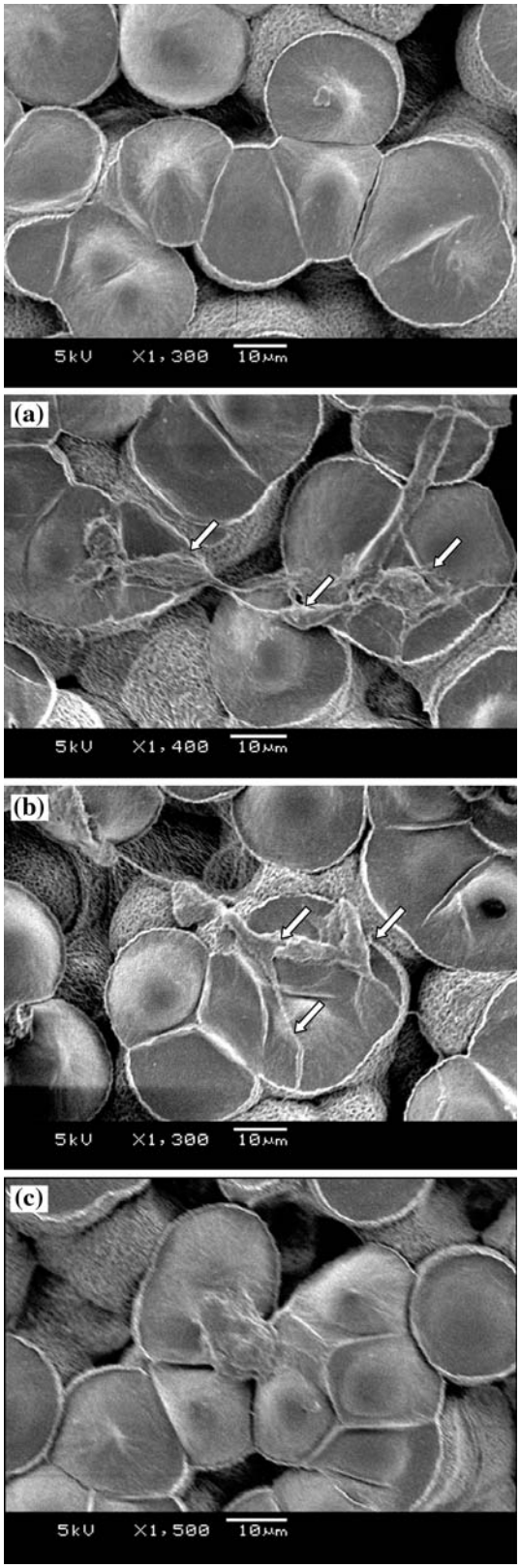

Fig. 6 1: Shows the microstructure of a PLGA film. 2(**a**) and (**b**) Shows C17.2 cells attached on the PLGA film scaffold. The cells are found to attach on the surface of the film. The neurite was seen to penetrate into the film, (**c**) shows the undifferentiated C17.2 cell scaffold with undifferentiated cells on the aligned microfiber scaffold is seen in Fig. 5.2(c).

In the PLGA film scaffold Fig. 6.1, the film was found to have a porous structure. Hence most of the cells passed through the porous structure. Some of the differentiated cells were seen on the surface of the porous film, Fig. 6.2(a) and (b). The neurites of the differentiated neuron were seen to penetrate into the film scaffold. Fig. 6.2(c) shows the reference film scaffold with undifferentiated cells on the PLGA film scaffold.

Hence depending on the application, the above mentioned scaffolds can be used for various tissue engineering purposes. Work on oesteoblasts cultured on PLGA foams has been reported earlier [20, 21]. However, no study on NSC's culture on polymer scaffold has been reported so far.

Conclusions

The present study proved the feasibility of PLGA 10:90 biodegradable polymer as a suitable material for C17.2 nerve stem cell culture, that can be used for nerve tissue engineering purposes. The cells attached and differentiated on the four types of scaffolds studied. All the scaffolds maintained their structural integrity. The cells were attached more on the surface in the case of PLGA nanofiber scaffold. The cells were distributed randomnly on the surface. The differentiated neurite took a randomn orientation on the scaffold. In the microbraided and aligned microfiber scaffolds, the cells were found to be aligned along the direction of the fiber. The neurites attached and grew along the direction of the fiber. Thus, on the aligned microfiber scaffolds aligned orientation of the cells was achieved. In the polymer film scaffold, the cells on the surface were less compared to the nanofiber scaffold due to the porous nature of the film. However, cell adhesion and differentiation of the nerve stem cells was observed in all the four types of scaffolds studied. These cell scaffold constructs can be used as cell carriers for nerve tissue engineering purposes. Hence, the present study proved the feasibility of PLGA (10:90) polymer as a potential candidate for repair or regeneration in the CNS.

References

1. Bandtlow CE (2003) Exp Gerontol 38:79
2. Woerly S, Plant GW, Harvery AR (1996) Biomaterials 17:301

3. Gurgom RD, Bedi KS, Nurcombe V (2002) J Clin Neurosci 9(6):613
4. Thomson RC, Shung AK, Yaszemski MJ, Mikos AG (2000) In: Lanza RP, Langer R, Vacanti JP (eds) Principles of tissue engineering. Academic Press, San Diego, p 251
5. Kenawy E-R, Bowlin GL, Mansfield K, Layman J, Simpson DG, Sanders EH, Wnek GE (2002) J Control Release 81:57
6. Yoshimoto H, Shin YM, Terai H, Vacanti JP (2003) Biomaterials 24:2077
7. Kenawy E-R, Layman JM, Watkins JR, Bowlin GL, Matthews JA, Simpson DG, Wnek GE (2003) Biomaterials 24:907
8. Zong X, Kim K, Fang D, Ran S, Hsiao B, Chu B (2002) Polymer 42:4402
9. Snyder EY, Deitcher DL, Walsh C, Arnold-Aldea S, Hartwieg EA, Cepkp CL (1992) Cell 68:33
10. Snyder EY, Yoon C, Flax JD, Macklis JD (1997) Proc Natl Acad Sci, USA 94:11663
11. Lynch WP, Sharpe AH, Snyder EY (1992) J Virol 73:6841
12. Holmes TC, Lacalle SD, Su X, Liu G, Rich A, Zhang S (2002) Proc Natl Acad Sci USA 97:6728
13. Freire E, Gomes FCA, Linden R, Neto VM, Coelho-Sampaio T (2002) J Cell Sci 115:4867
14. Miller C, Jeftinija S, Mallapragada S (2001) Tissue Eng 7:705
15. Jucker M, Kleinman HK, Ingram DK (1991) J Neurosci Res 28:507
16. Rajnicek AM, Britland S, McCaig CD (1991) J Cell Sci 110:2905
17. Ceballos D, Navarro X, Dubey N, Wendelschafer-Crabb G, Kennedy WR, Tranquillo RT (1999) Exp Neurol 158:290
18. Saneinejad S, Shoichet MS (2000) J Biomed Mater Res 50:465
19. Wojciak-Stothard B, Curtis ASG, Monaghan W et al (1995) Cell Motil Cytoskeleton 31:147
20. Terai H, Hannouche D, Ochoa E, Yamano Y, Vacanti JP (2002) Mater Sci Eng C 20:3
21. Goldstein AS, Zhu G, Morris GE, Meslenyi RK, Mikos AG‘ (1999) Tissue Eng 5:421