

In vitro cell viability of PHBV/PLGA nanofibrous membrane for tissue engineering

Mohd Izzat Hassan ^a, Naznin Sultana ^{b, *}

^a Faculty of Biosciences & Medical Engineering, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia

^b Medical Academy, Prairie View A&M University, TX77446, Texas, USA

* Corresponding author: nsultana@pvamu.edu; naznin@biomedical.utm.my

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Abstract

In recent decades, tissue engineering has emerged as an alternative solution to improve the quality of life for patients who suffer from diseases involving loss of tissues. The development of a biocompatible tissue engineering scaffold using nanofibrous membrane has garnered increasing interest in biomedical applications due to its similarity of structural property to the extracellular matrix, which is essential for cell growth. Thus, this study was conducted to fabricate a nanofibrous membrane with small diameter structure using a polymer blend of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) and poly(lactic-co-glycolic acid) (PLGA) and through the electrospinning technique, and to evaluate its biocompatibility with fibroblast cells. The nanofibrous structure was optimized using three different ratios (25:75; 50:50; 75:25) of PHBV/PLGA polymer. Characterization of the nanofibrous membrane was done using a scanning electron microscope (SEM), attenuated total reflection - Fourier transform infrared spectroscopy (ATR-FTIR), and water contact angle (WCA). Biocompatibility evaluation was done by culturing the nanofibrous membrane with human skin fibroblast cells (HSF), then measuring cell viability using MTT and live/dead assays. Results indicated that the nanofibrous membrane obtained a homogeneous morphology at 50:50 ratio with a fiber diameter range of 200 – 1000 nm, had a minimum contact angle of $106.90 \pm 4.42^\circ$, and good cell viability with HSF. Analyses from the SEM analysis and live/dead assays showed that the cells had proliferated after 7 days. Findings from this study suggest that PHBV/PLGA nanofibrous membrane can be used as a biocompatible artificial scaffold in tissue engineering application.

Keywords: Electrospinning, nanofibers, PHBV, PLGA, cell viability

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INTRODUCTION

Tissue engineering is very important in regenerative medicine because it applies an advanced field of engineering, biology, chemistry, and medicine to produce a substitute structure called scaffold for regeneration of tissue loss. The purpose of tissue engineering is to overcome the limitation in organ transplantation. The engineering of scaffolds is one of the strategies to provide temporary support for damaged biological tissues. An ideal scaffold material should be able to biologically, chemically, and mechanically mimic the living tissue (Okamoto *et al.*, 2013; Sala *et al.*, 2013). Currently, there are many techniques to fabricate tissue engineering scaffold such as freeze drying/phase separation (Lari *et al.*, 2017; Guo *et al.*, 2017), particulate leaching (Scaffaro *et al.*, 2016), solution casting (Sangsanoh *et al.*, 2017), gas foaming (Costantini *et al.*, 2016), and electrospinning (Izzat *et al.*, 2017). Electrospinning is one of the techniques used to create a nano-scale fibrous structure which is similar to the natural extracellular matrix (ECM) found in human. As ECM provides the environment for living cells to grow, therefore a nanofibrous scaffold would be able to provide similar function by giving structure to the tissue and encouraging cell proliferation (Ndreu *et al.*, 2008).

Electrospinning uses an electrostatic field to produce fibrous materials. Typical set-up of electrospinning needs a capillary, a high voltage power supply, and a grounded collector. During the electrospinning process, a polymeric solution will be forced out from the capillary tip by applying a potential difference/voltage between the capillary and collector, resulting in the break-up of liquid jet onto the

collector. Variables such as polymer concentration or viscosity of the solution, flow rate of the syringe pump, voltage applied, tip-to-collector distance, and ambient room condition can affect the final morphology of the nanofibrous membrane (Tamayol *et al.*, 2013).

In the development of tissue engineering scaffold, choosing the right biomaterial, such as a biodegradable polymer, is important. Produced from microorganism fermentation, poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) is one of the viable materials for tissue engineering scaffold. It is biocompatible, biodegradable, and non-toxic to living cells, making it favorable for biomedical applications. Moreover, the degradation waste of PHBV in the body is a normal constituent of the blood (Bai *et al.*, 2015). On the other hand, biodegradable polyester poly(lactide-co-glycolide) (PLGA) has been extensively used in drug delivery, surgery, and tissue engineering scaffold applications. It has been approved by the U.S. Food and Drug Administration for biomedical applications in the human body. In addition, the combination of lactic acid and glycolide acid in PLGA can vary in ratio. Thus, it can be used in different tissue applications, such as skin and bone, at a suitable degradation rate (Haider *et al.*, 2014).

In this study, the objectives were to fabricate PHBV/PLGA nanofibers and evaluate its viability with fibroblast cells. The nanofibrous scaffold was fabricated by blending PHBV and PLGA polymers using an electrospinning technique. Blending two polymers may improve the characteristics of nanofibers such as the morphological and mechanical properties of its structure, and chemical and biological properties of its surface. Characteristic evaluations of the nanofiber scaffold are needed to determine the optimal nanofibrous

membrane for cell growth. The morphology characteristics, such as diameter and porosity, of nanofibers have significant influences on cell attachment and proliferation (Chen *et al.*, 2007; Guimarães *et al.*, 2010). Surface hydrophilicity and hydrophobicity of nanofibers are also important in cell attachment as cells are more prone to attach to hydrophilic surfaces. Properties of the nanofibrous scaffold fabricated in this study were evaluated using the scanning electron microscope (SEM), water contact angle measurement, and attenuated total reflection Fourier transform – infrared spectroscopy (ATR-FTIR). Finally, the cell viability of PHBV/PLGA nanofibers was evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and live/dead assays.

EXPERIMENTAL

Materials

PHBV polymer (PHV content 12 mol %), PLGA polymer (lactide:glycolide = 85:15) with a molecular weight of 50,000-75,000, and analytical grade Trifluoroethanol (TFE) solvent were purchased from Sigma Aldrich, USA.

Electrospinning of nanofibrous membrane

Pure polymer solutions were prepared at different concentrations, such as 20, 24, and 26% (w/v) for optimization. PHBV and PLGA blends were prepared at 26% (w/v) concentration at different blending ratios (25:75, 50:50, 75:25). The solution was dissolved by magnetically stirring in TFE solvent at 50°C. The dissolved solution was transferred to a 5 mL syringe with 21G blunt-needle, which was then secured to the electrospinning machine equipped with a syringe pump (NE-300, New Era Pump Systems Inc., USA) and a voltage power supply. The electrospinning was set up in a horizontal position with a voltage supply of 20kV, a flow rate of 3mL/h, and a tip-to-collector distance of 10cm. The humidity of the room was at 50 – 60 % relative humidity. The collected samples were kept in a desiccator prior to characterization.

Characterization of PHBV/PLGA nanofibrous membrane

Scanning electron microscope (SEM)

A small section of each sample was cut and attached on to the stub. The morphology of nanofiber was observed under the SEM (Hitachi TM3000, Japan) at an accelerating voltage of 15 kV.

Energy dispersive X-ray spectroscopy (EDX)

Energy dispersive X-ray (EDX) was used to identify the elements in the nanofiber. The pore size and diameter of the nanofiber were measured using an image analysis software (ImageJ, NIH, USA). Diameters of at least 50 fibers were measured and then averaged.

Water contact angle

The surface wettability of the nanofibrous membrane was analyzed using a contact angle instrument (VCA-optima, USA) by dropping 2 µl of deionized water onto the membrane. Five water contact angles were taken at different locations and then averaged.

Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

Attenuated total reflection Fourier transform infrared spectra (Perkin-Elmer Spectrometer, MA, USA) of the nanofibrous membrane was investigated in the region of 4000 – 500 cm⁻¹ by clamping the sample on the ATR diamond crystal.

Sterilization of nanofibers for cell culture

The nanofibrous scaffolds were first cut into circular discs to fit 24 well plates. The scaffold discs were exposed to ultraviolet for 2 hours, then washed with phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin. Lastly, the scaffolds were immersed in a medium before cell seeding.

Cell culture

Human skin fibroblast cells (HSF 1184, ECACC, UK) were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% Pen-Strep in a CO₂ incubator (37°C, 5% CO₂) with a humidified atmosphere of 95%. DMEM was replaced every three days. When HSF cells reached 80-90% confluence, they were immersed/detached with Accutase and counted using a hemocytometer (Counting chambers, Neubauer-improved, Hirschmann).

MTT assay

HSF cells at a density of 1x10⁴ cells/well were seeded on each type of scaffold in 24 well plates. After 72 hours, the medium was replaced with 100 µl medium and 10 µl MTT. Then, the plate was further incubated in the dark for 4 hours. Dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystal of MTT. The absorbance of MTT in 96 well plates was measured at a wavelength of 490 nm using a microplate reader (Multiskan FC, Thermo, Waltham, MA, USA).

Live/dead assay

For live/dead viability assessment, HSF cells were seeded on the scaffold samples at a density of 1x10⁵ cells/well. The medium was changed every three days. After seven days, they were stained with the Live/dead Viability/Cytotoxicity Assay (InvitrogenTM, USA) according to the manufacturer's protocol to mark the live and dead cells attached to the surface. Viability of the cell was visualized/observed using an inverted fluorescence microscope (Axiovert S100, Carl Zeiss, Germany).

Cell morphology

For cell morphology, cells were seeded on the scaffold at a density of 1x10⁵ cells/well in 24 well plates. After seven days, the nanofibrous scaffolds were transferred to another 24 well plates and the medium was removed. The scaffolds were then washed with PBS and fixed with 4% glutaraldehyde in PBS for 1 hour at room temperature. After fixing, the scaffolds were rinsed with PBS and gradually dehydrated in 30%, 50%, 70%, 95% and 100% of ethanol for two minutes at each stage, then air-dried before putting into a desiccator. Next, cells on the scaffold were observed using SEM in non-charging mode. All data were presented as mean ± standard deviations.

Statistical analysis

For statistical analysis, the data were tested using two-tailed Student's t-test with a significant level of p < 0.05.

RESULTS AND DISCUSSION

Optimization of pure PHBV and pure PLGA nanofibrous membrane

Before mixing two different polymers, pure PHBV and pure PLGA were dissolved in trifluoroethanol at different concentrations of 20, 24, and 26% (w/v). Throughout the whole electrospinning process, the flow rate of the syringe pump, the distance between the needle-tip and aluminium collector, and a voltage applied, were set as constant while concentrations of the electrospun solution were varied according to blending compositions. Micrographs of the electrospun nanofibers observed under SEM are shown in Fig. 1 for pure PHBV and Fig. 2 for pure PLGA. At 20% (w/v), only bead formation was observed. After increasing the concentration to 24% (w/v), some fibers were produced, leading to the formation of beaded fibers. The optimal solution concentration was determined at 26% (w/v) which produced fine electrospun nanofibers. A concentration that was higher than 26% (w/v) was not considered in the blending preparation since many researchers reported the effect of increased fiber diameter with the increase of polymer concentration (Cadafalch Gazquez *et al.*, 2017). However, such studies could be explored in the future to prove the effect of higher concentration on the diameter of fibers. Previously, increased in fiber diameter due to higher concentration has been determined for both PLGA and PHBV polymers (Nguyen *et al.*, 2015; Tong *et al.*, 2015).

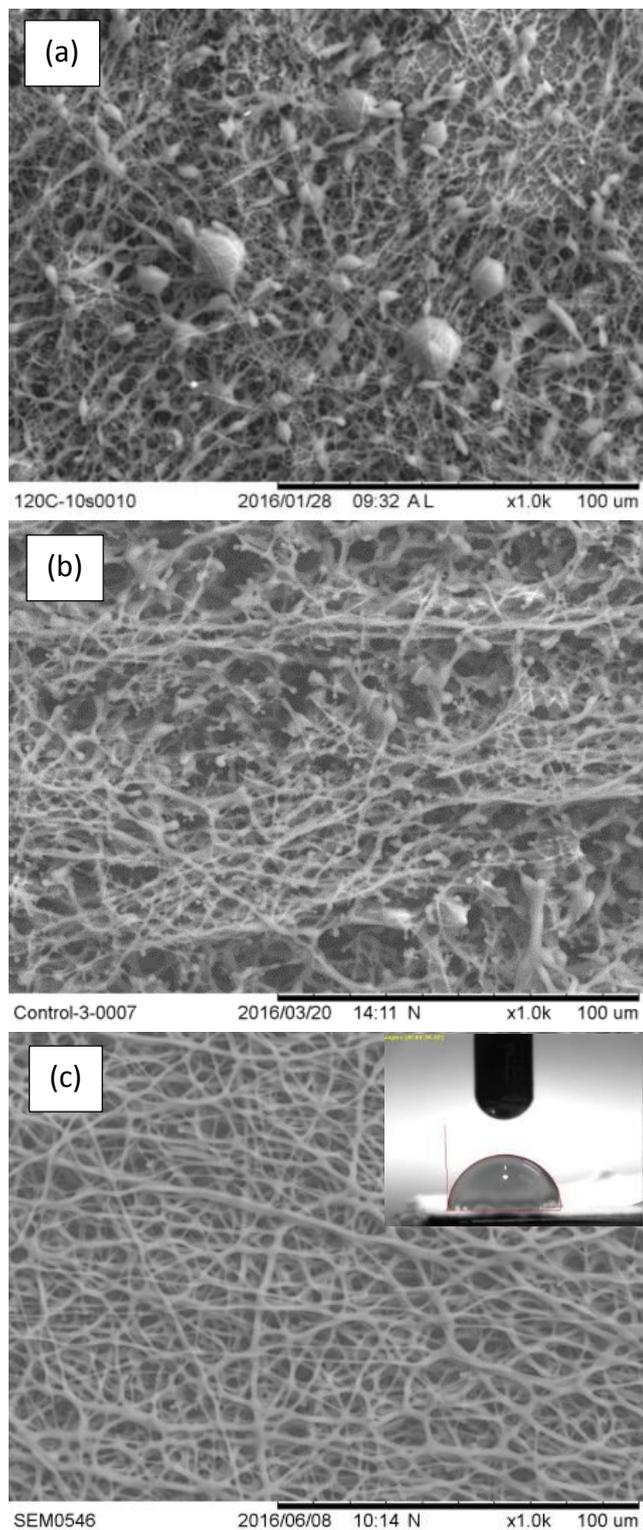


Fig. 1 SEM micrographs of pure PHBV electrospun fibers at a concentration of (a) 20, (b) 24, and (c) 26 % (w/v) with water contact angle image.

In addition, fibers with higher diameter are less favorable for cell attachment and proliferation. In one study, Chen reported that the cell adhesion and proliferation decreased when the average fiber diameter was changed from 428 nm to 1051 nm (Chen *et al.*, 2007).

Morphological analysis of PHBV/PLGA nanofibrous membrane

After the fabrication of nanofibers at different ratios of (a) 75:25, (b) 50:50, (c) 25:75 PHBV/PLGA polymer, the morphological analysis of the nanofibrous membrane scaffold was investigated using SEM and

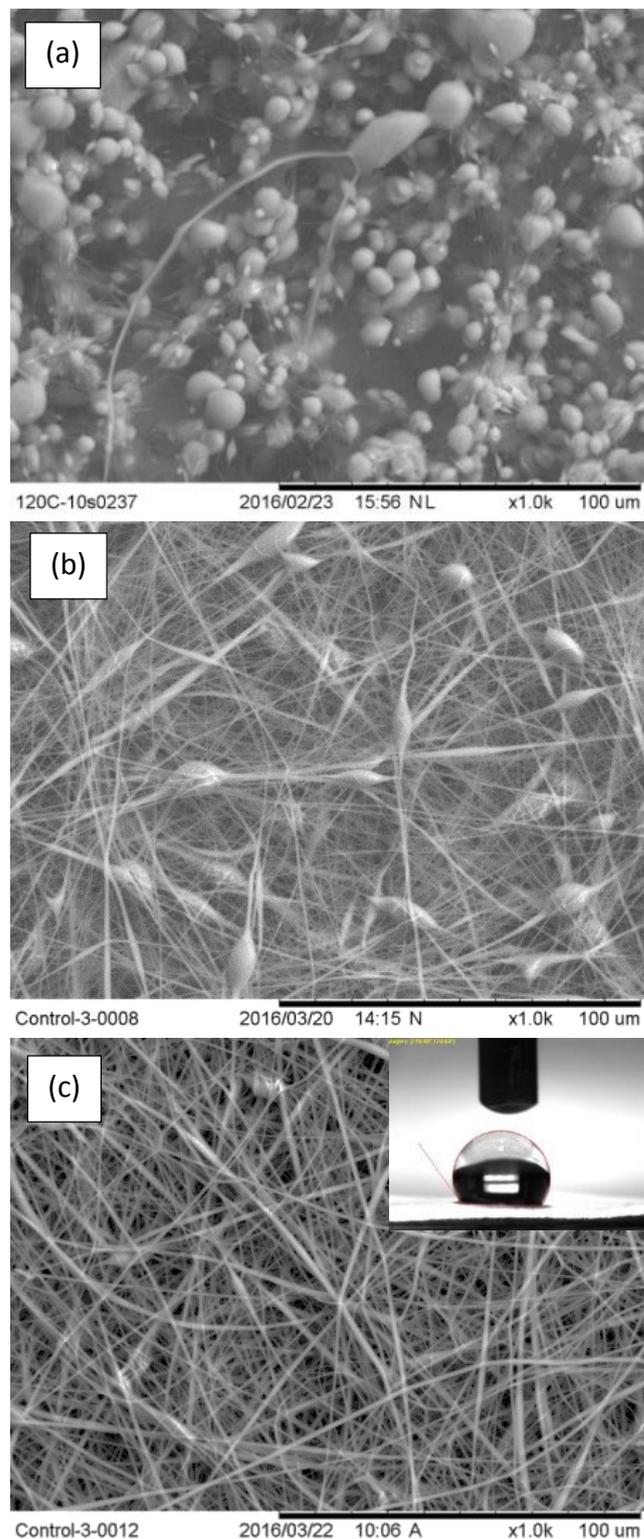


Fig. 2 SEM micrographs of pure PLGA electrospun fibers at a concentration of (a) 20, (b) 24, and (c) 26 % (w/v) with water contact angle image.

water contact angle equipment. Results are shown in Fig. 3, Fig. 4, and Table 1.

The nanofiber structures at three different ratios were obtained by optimizing the polymer solution at 26% (w/v) concentration and dissolving it in TFE solvent in order to obtain a non-woven nanofibrous structure as shown in the SEM micrographs (Fig.3). Below the optimized concentration, the viscosity of the polymer solution was low and insufficient to produce fine fibers, resulting in either no formation of nanofibers (i.e. solution droplet) or fibers with many bead defects. Blending two different polymers is a challenging process since only

selected solvents can dissolve the polymers. Diameters and pore sizes from the SEM micrographs were obtained using ImageJ software. The average fiber diameter increased with the increase of PHBV content in the polymer blend PHBV/PLGA ratio. At the 75:25 ratio, the nanofibers had the highest diameter of 673.51 ± 146.61 nm but with a smaller pore size of $13.68 \pm 7.69 \mu\text{m}^2$.

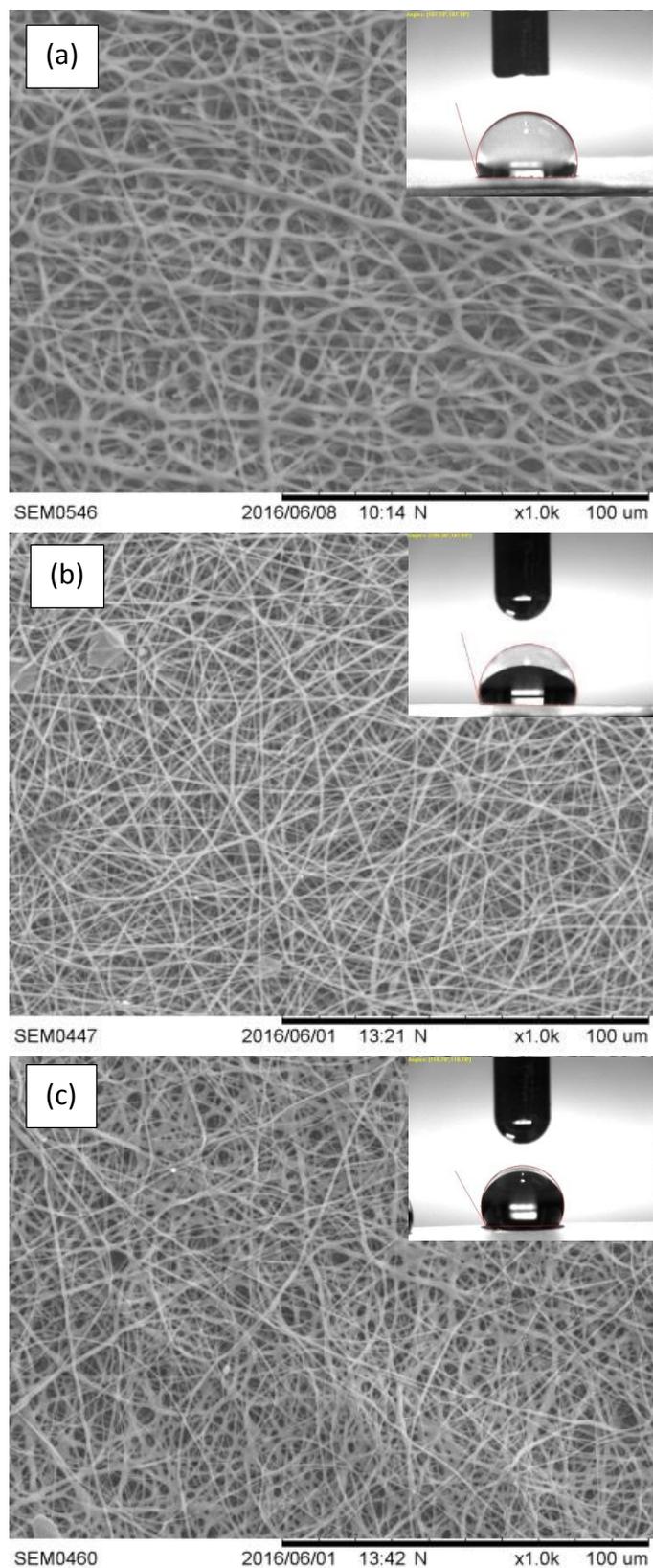


Fig. 3 SEM micrographs of PHBV/PLGA nanofibrous membrane electrospun at 26% (w/v) with water contact angle images at the ratio of (a) 75:25, (b) 50:50, and (c) 25:75.

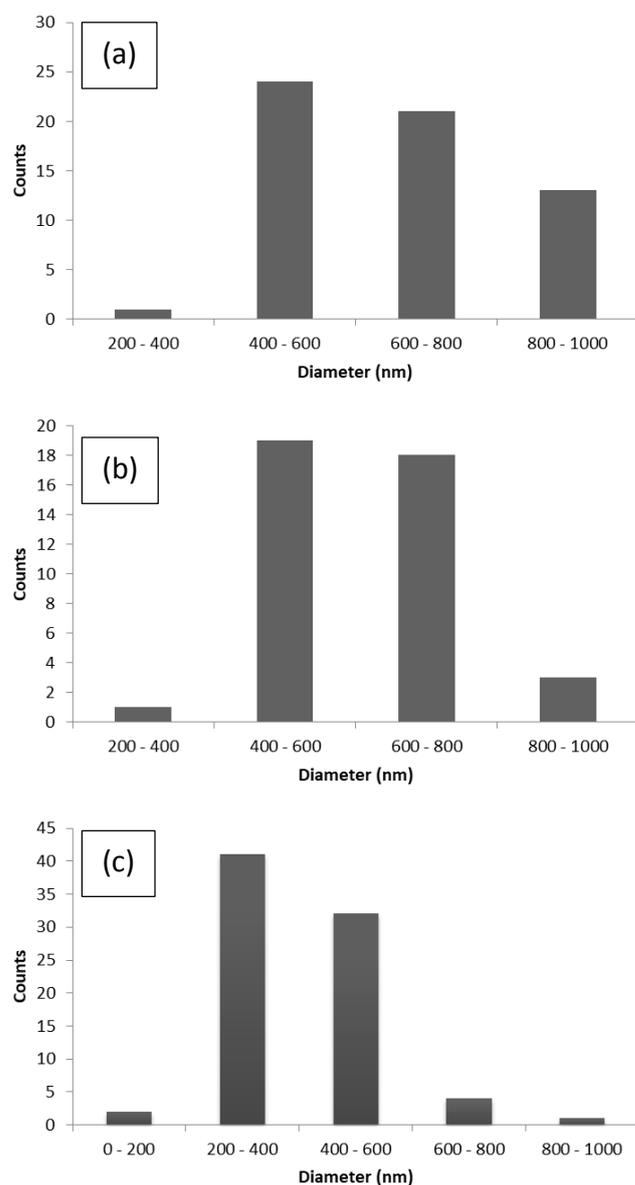


Fig. 4 Diameter distribution of nanofiber at the ratio of (a) 75:25, (b) 50:50, and (c) 25:75.

Table 1 Average fiber diameter, pore size, and water contact angle of PHBV/PLGA nanofibrous membrane at different ratios.

PHBV/PLGA Ratio	Average fiber Diameter (nm)	Pore size (μm^2)	Contact angle ($^\circ$)
25:75	411.89 ± 127.33	20.63 ± 11.82	116.90 ± 4.34
50:50	613.17 ± 121.69	21.73 ± 10.45	106.90 ± 4.42
75:25	673.51 ± 146.61	13.68 ± 7.69	107.67 ± 0.54

Water contact angle analysis

The wettability of the membrane was determined by measuring the contact angle of water droplet on its surface. Generally, the hydrophilic surface has a lower contact angle than 90° and the hydrophobic surface has a higher contact angle than 90° . Basically, an adequate hydrophilic surface with a low water contact angle is desirable for cell attachment (Kim et al., 2006; Vasita et al., 2010). For pure PHBV as shown in Fig. 1(c), the lowest water contact angle was at $93.65 \pm 4.10^\circ$ which is close to the hydrophilic value of 90° . Meanwhile, pure PLGA as shown in Fig. 2(c) had the highest water contact angle of $120.21 \pm 4.65^\circ$ which reflects a hydrophobic property. After the blending of fibers as shown in Fig. 3 and Table 1, the lowest contact angle was measured at 50:50 ratio with a value of $106.90 \pm 4.42^\circ$. There was no significant difference

between the contact angle between the 50:50 and 75:25 ratios. The small increment of contact angle value observed in the latter ratio may be due to the higher average of fiber diameter. Thus, the nanofibrous membrane at this ratio (50:50) was chosen for biocompatibility evaluation with fibroblast cells.

Attenuated total reflection Fourier transform infrared spectroscopy

The ATR-FTIR was used to study the interaction and type of bonding present between the two polymers.

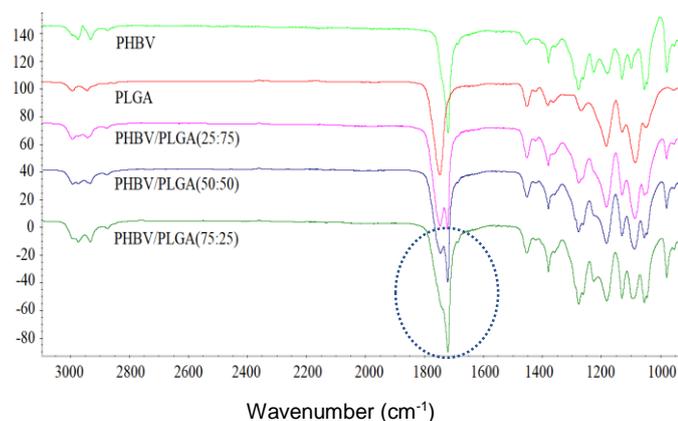


Fig. 5 ATR-FTIR spectra of pure PHBV and PLGA, and blended PHBV/PLGA nanofibrous membranes.

Fig. 5 shows the FTIR spectra for pure PHBV and PLGA, and blended PHBV/PLGA nanofibrous membranes. For pure PHBV, the observed C=O stretching vibration at 1720 cm^{-1} ; C=O stretching bands at 1278 cm^{-1} and 1054 cm^{-1} ; C-H stretching bands at 2974 and 2935 cm^{-1} ; and C-H bending vibrations at 1453 and 138 cm^{-1} . The obtained results presented the typical peaks of PHBV (Ai et al., 2011). For pure PLGA, the observed C=O stretching at 1750 cm^{-1} , C-O bands at 1085 - 1450 cm^{-1} , and C-H stretching vibration at 2820 cm^{-1} , presenting the typical peaks of PLGA (Khalil et al., 2013). The major spectral change observed in peaks between 1600 - 1800 cm^{-1} wavelength indicated the existence of molecular interaction in the PHBV/PLGA polymer blends with different blending ratios. In the case of PHBV/PLGA at 25:75 ratio, there were two peak appearances in the carbonyl region between 1600 - 1800 cm^{-1} which represented the characteristics of pure PLGA and pure PHBV. Higher intensity of a characteristic peak of PHBV was observed for 50:50 and 75:25 blend where higher content of PHBV had the highest intensity.

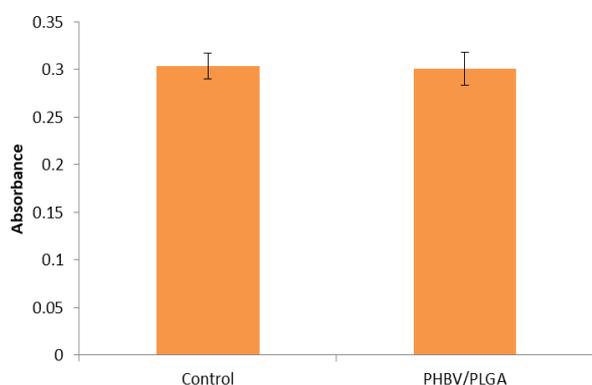


Fig. 6 MTT assay of HSF on PHBV/PLGA nanofibrous membrane compared to control TCP.

Biocompatibility of PHBV/PLGA nanofibrous membrane

Biocompatibility of a scaffold is important for its application in tissue engineering to ensure no undesirable effect is elicited in the host tissue. Although PHBV and PLGA polymers are known to be biocompatible, it is compulsory for the fabricated materials to follow and comply with the ISO standard 10993. In this study, the

biocompatibility of the nanofibrous membrane scaffold was evaluated using *in vitro* culture with HSF cells after three days, and the results of the MTT absorbance reading were compared to the tissue culture plate (TCP) denoted as a control in Fig. 6. The MTT assay is based on the reduction of yellow tetrazolium salt to purple formazan crystals by the dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed was proportional to the number of viable cells. The results obtained showed no significant difference ($p > 0.05$) between PHBV/PLGA nanofibrous membrane and TCP. The calculated relative viability of HSF cells to the control TCP was 95% cell viability.

Qualitative observation of HSF cells in contact with the nanofibers was evaluated using live/dead assay and SEM after seven days of culture. Through live/dead assay, the live cells were stained green while dead cells were stained red. Fig. 7 (a) shows the merged image of both live and dead cells. Since most of the live cells covered the surface of the membrane, only green color can be seen in the merged image. Through the enzymatic conversion of the virtually nonfluorescent cell-permeant calcein-AM to the intensely fluorescent calcein, the living cells were stained green (i.e. excitation/emission $\sim 495\text{ nm}/\sim 515\text{ nm}$). Dead cells were stained red (i.e. excitation/emission $\sim 495\text{ nm}/\sim 635\text{ nm}$) as EthD-1 has entered into the damaged membranes of cells and bound to the nucleic acids. From Fig. 7(a), viable cells calculated from at least three different surface area indicated that more than 95% of live cells were in contact with the membrane.

The SEM micrograph of the nanofibers, which have undergone seven days of cell culture and further fixation, presented in Fig. 7(b) shows the morphology of cells spreading onto the membrane interface. A few of HSF cells had normal spindle-like morphology whereas most of the cells formed multilayered construct that covered most of the membrane surface after seven days of culture, as illustrated in Fig.7(a) and (b). The overall analysis confirmed that the PHBV/PLGA membrane was biocompatible. However, further evaluation of this nanofiber blend on other aspects of biocompatibility such as cells proliferation, cells infiltration and, biodegradability is required to be performed for the successful application for tissue regeneration.

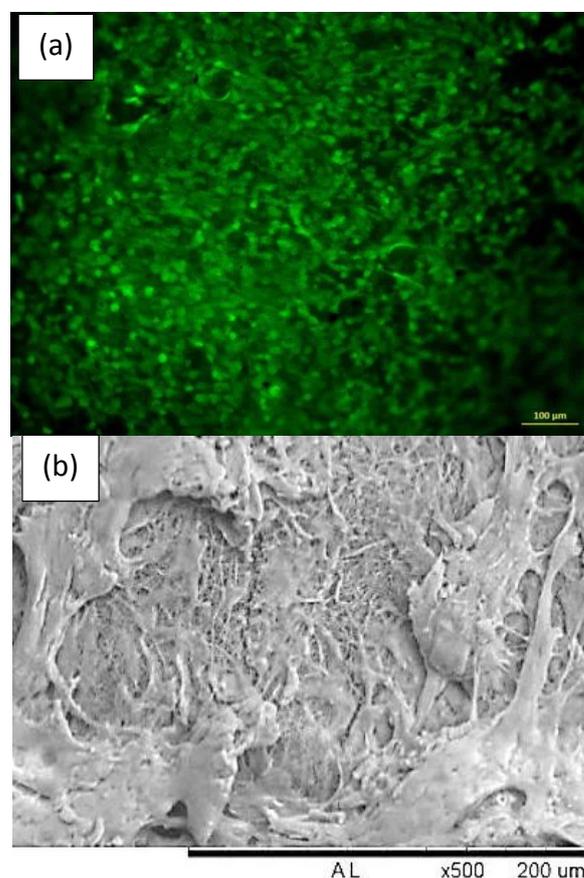


Fig. 7 (a) Live/dead assay and (b) SEM micrograph of HSF proliferated on PHBV/PLGA nanofibrous membrane after seven days of culture.

CONCLUSION

In conclusion, polymer concentration plays an important role in the formation of fine nanofiber from pure PHBV and PLGA polymers. At an optimal concentration of 26% (w/v), PHBV/PLGA nanofibrous membrane was fabricated using electrospinning technique at different blending ratios (25:75; 50:50; 75:25). The membrane with a 50:50 ratio chosen for the viability assessment had a diameter of 613.17 ± 121.69 nm, the lowest contact angle of $106.90 \pm 4.42^\circ$, and a better pore size of $21.73 \pm 10.45 \mu\text{m}^2$. Interactions between PHBV and PLGA polymers after blending were observed in the peaks of the ATR-FTIR spectra. Results from the MTT and live/dead assays, along with the observation of SEM indicated good biocompatibility of the fabricated nanofibrous membrane. This suggests that the membrane has the potential to be applied as a tissue engineering Scaffold.

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