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CHARACTERIZATION OF NANOFIBROUS SCAFFOLDS AS A SUBSTRATE FOR DRUG DELIVERY

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Abstract

Biomaterials play a crucial role in regenerative medicine strategies by serving as 3D frameworks for cellular attachment and proliferation. Nanofibrous scaffolds characterized by high surface-to-volume ratio, high porosity can be fabricated for sustained release of various bioactive materials and drugs. Electrospinning represents an attractive technique for the fabrication of polymeric biomaterials into nanofibers. In this study the nanofibrous scaffolds of three different polymer composites poly-ε-caprolactone (PCL)-collagen (COLL), PCL-polylactic-co-glycolic acid (PLGA), and PCL alone were successfully generated in the form of sheets using the electrospinning setup and subsequently characterised. Nanofibrous scaffolds were incorporated with tetracycline and vancomycin and the in vitro release rate was measured spectrophotometrically. FTIR analysis revealed the presence of functional groups such as C=O, COC, C-H of the polymers and amide I, amide II and amide III bonds of collagen. The melting temperature of PCL-COLL and PCL-PLGA scaffolds was found to be 56.81 °C and 57.43 °C respectively. It was found that PCL-COLL and PCL-PLGA nanofibrous scaffolds were biocompatible. Drug release studies showed that the drug can be effectively encapsulated into the nanofibrous scaffold with initial burst followed by sustained release of the drug. The rate of release was found to be similar irrespective of the type of polymer used and drug encapsulated. These antibiotic loaded electrospun nanofibrous scaffolds could be effectively used as wound dressing materials.

Introduction

Tissue engineering is a new approach for the reconstruction and/or regeneration of lost or damaged tissue [1]. It is a multidisciplinary field that applies the principles of engineering and life science by combining synthetic and living components in appropriate configurations and environmental conditions [2, 3]. With respect to all organs in the human body, the extracellular matrix (ECM) is a network of various complex proteins such as collagen, laminin, proteoglycans, integrins etc. and plays a pivotal role in controlling cell behavior [4]. Biopolymers or polymers derived from living organisms, such as collagen is of great interest in tissue engineering. Collagen is the most widely found protein in mammals (25% of total protein mass) and is the major provider of strength to tissue. The collagen networks form a highly organized, three-dimensional architecture which interacts with other proteins. The collagen obtained from various sources like bovine skin, fish skin, rat tail tendons etc. could be used as an ideal scaffold which can be used for many tissue engineering applications by serving as a matrix for cellular in growth, proliferation and new tissue formation in three dimensions [5]. Scientific investigations involving collagen have inspired tissue engineering and design of biomaterials, since collagen fibrils and their networks primarily regulate and define most tissues. Scaffold generated by this way should mimic the structure and biological function of native extracellular matrix as much as possible [6].

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Along with naturally obtained proteins different types of synthetic biopolymers, biodegradable polyesters, such as poly (glycolic acid), poly (lactic acid), poly (hydroxyl butyrate), and poly (ε-caprolactone) as well as their copolymers have been widely used for multiple biomedical applications [7-11]. Among these biodegradable polymers, PCL is of great interest due to its soft- and hard-tissue compatibility and ease of processing. A combination of natural and biodegradable synthetic polymers could lead to an ideal scaffold for broad range of tissue engineering applications. There are several methods available for the generation of 3D scaffolds such as solid freeform fabrication techniques (three-dimensional printing (3D-P), fused deposition modeling (FDM), selective laser sintering (SLS)). The three-dimensional nanofibrous environment promotes in vivo-like cellular phenotypes and promote tissue morphogenesis. Nanofibrous scaffolds are ideal for controlling the cell functions because their dimensions are similar to components in the ECM and mimic its fibrillar structure, providing essential cues for cellular organization, survival and function. Nanofibrous scaffolds have inherently high porosities and surface-area-to-volume ratios whilst also offering a wide variety of topographical features to encourage cellular adhesion and proliferation. The process of utilizing electrostatic forces to make synthetic fibers is known as electro spinning. This utilizes high voltage source to inject charge of a certain polarity into the polymer solution, which is then accelerated towards a collector of opposite polarity. The fiber jet travels through the atmosphere allowing the solvent to evaporate, thus leading to the deposition of solid polymer fibers on the collector. Fibers produced using this process typically has diameters in the order of few micrometers down to the tens of nanometers [12]. Electrospinning helps in creating ECM analogue scaffolds composed of nanoscale fibers, with very high surface area to volume ratio to support cell growth and infiltration. The morphological similarities between the nanofibrous structures and the native ECM are believed to improve cellular response and overall biocompatibility. Nanofibers produced by electrospinning have several potential advantages in filtration, protective clothing and biological applications like tissue engineering, scaffolds and drug delivery devices [13]. Nanofibrous scaffolds encapsulated with an anti infective agents and releasing it at required phase of wound healing would be ideal for wound dressing. Also nanofibrous scaffolds encapsulated with anti infective agents could be effectively used in case of wounds that are potentially at the risk of infection. Drugs encapsulated in nanofibers synthesized using single step electrospinning provide an initial burst release and this may impair therapeutic efficiency [14-18]. In the present study we have validated composite nanofibrous scaffold which could serve as a wound dressing material capable of controlled release of antibiotics. Tetracycline is a broad spectrum antibiotic that mediates anti inflammatory action [19]. Vancomycin is a tricyclic glycopeptide antibiotic which inhibits bacterial cell wall synthesis and has been widely used in the treatment of methycillin resistant *Staphylococcus aureus* in patients intolerant to beta lactam antibiotics [20]. In this study we made an attempt to characterize composite nanofibrous scaffolds and encapsulate tetracycline and vancomycin antibiotics in PCL and PCL-Collagen composite and studied the *in vitro* drug release rate followed by biocompatibility studies of the nanofibrous scaffolds.

Materials and Methods

Materials

Poly-ε-caprolactone (PCL), polylactic-co-glycolic acid (PLGA(75:25), resomer RG752S), 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), collagen type I (COLL, isolated from fish skin), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reagent, sigma), isopropanol (loba chemie), phosphate buffered saline (PBS), tetracycline (piramal India limited), vancomycin (VHB mediscience limited), Dulbeccos minimal essential medium(DMEM), pencillin/streptomycin (Sigma), Trypsin (Sigma), and distilled water.

Methods

Generation of nanofibrous scaffolds

10% PCL and collagen type I solutions in the ratio of 75:25 were mixed and stirred for 72 hrs and subsequently loaded into electrospinning setup. Similarly a mixture of PCL and PLGA in the ratio of 75:25 was also prepared. A sterile 2ml syringe with a 21G needle was used. It was ensured that the needle was blunt; the polymer solution was

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loaded into the syringe. The syringe was then placed inside the electrospinning apparatus and the tip of the needle was firmly placed within the voltage clamp. The flow rate was set to 1 ml/hr and the voltage to 22KV**.** Random nanofibrous scaffolds were collected on an aluminum foil and were carefully removed in the form of a sheet.

Characterization of nanofibrous scaffolds

Functional groups of the composite nanofibers were characterized using Fourier Transform Infrared (FTIR) spectrometer (Perkin-Elmer, USA). The nanofibrous scaffolds were cut into small pieces, grinded with KBr and pressed into pellets. Measurements were taken in a range between 4000-500 cm^{-1} with a resolution of 2 cm^{-1}. The thermal characteristics were determined using a differential scanning calorimetry (DSC) (Q-200 differential scanning calorimeter TA, instruments Co., USA). Samples were crimped in a standard aluminium pan and heated from 40 $^{\circ}C$ to 250 $^{\circ}C$ at a heating rate 10 $^{\circ}C$ per minute under constant purging of nitrogen at 20 ml/min.

In vitro cytotoxicity of nanofibrous scaffolds

PCL-Collagen (10%) and PCL-PLGA (15%) nanofibers collected on the glass cover slips were used to study the cytotoxity of the nanofibers. The cover slips with nanofibers were placed into the wells of 6-well plate, and 5 X 10^4 mouse embryonic fibroblasts were seeded. MTT assay was performed on the cells at 24, 48 and 72 hrs to monitor the rate of cell viability. Briefly 200 µl of MTT reagent was added to each well containing 2 ml of medium and incubated at 37 ºC for 3 hours. After incubation the medium is discarded and the insoluble dye is solubilised using 2.5 ml of acidic isoproponol, kept aside for 2 min and the absorbance was measured spectrophotometrically at wavelength of 570 nm.

In vitro drug release studies

During the preparation of PCL (10%) and PCL-PLGA (15%) polymer solutions, antibiotics tetracycline and vancomycin at a concentration of 50 mg/5 ml were added to the polymer solution. The solution was allowed to mix homogenously for 24 hrs and nanofibers were generated using electrospinning. The antibiotic encapsulated nanofiber scaffold was cut into small pieces weighing 10 mg and suspended in 2ml of PBS. The absorbance of the vancomycin solution was measured at 280 nm and tetracycline was measured at 358 nm spectrophotometrically to determine the concentration.. The absorbance was measured at different time intervals of 30 min, 60 min, 120 min, 240 min, 1440 min (1 day), 2880 min (2 days), 4320 min (3 days), 5760 min (4 days), 8640 min (6 days) and 10080 min (7 days).

Agar diffusion test

Mueller Hinton agar plates were prepared and S*taphylococcus aureus* was inoculated on to the plates by spread plate method. Drug loaded nanofibrous scaffolds were placed over the above plates and their positions were appropriately marked for reference. A control disc with only polymer was also placed similarly. The plates were incubated at 37 ^0C for 24 hrs and the zone of inhibition was measured to monitor the effect of drug on microbial growth.

Statistical analysis

The experiments were conducted in triplicate and averages were presented as mean ± standard deviation (SD). Standard curves were fit between measured absorbance values and standards of known vancomycin and tetracycline concentrations by least squares linear regression for all repetitions of the assay. Coefficients of determination (R^2) were calculated for each standard curve. All the statistical analysis was done by using Graphpad prism software.

Results and discussion

Generation of nanofibrous scaffolds

Bright field microscope images of electrospun nanofibrous scaffold from PCL-Collagen and PCL-PLGA showed continuous, bead free, smooth and randomly oriented fiber morphology (Figure 1B and 1C). It is also evident that PCL-PLGA electrospun fiber mats have interconnected pores (Figure 1C). Basically a scaffold should be porous in

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order to permit the infiltration of cells, oxygen and nutrients through it, thereby promoting cell migration and proliferation.

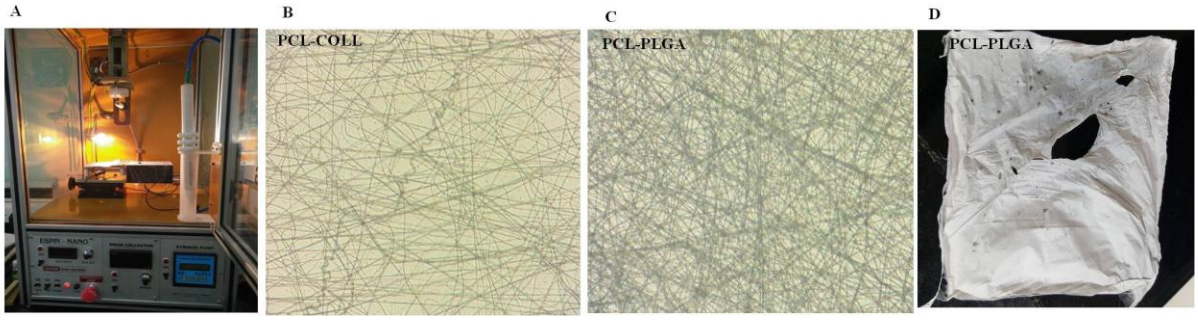

Figure 1: Morphology of nanofibers. Representative photomicrographs of A. Electrospinning setup B. Nanofibers generated from PCL-Collagen C. Nanofibers generated from PCL-PLGA D. Nanofibrous mat generated from PCL-PLGA.

Characterization of nanofibrous scaffolds

The functional groups present in the nanofibrous scaffolds were assessed by FTIR. PCL-COLL and PCL-PLGA scaffolds showed characteristic peaks corresponding to the functional groups of the polymer composites. FTIR analysis of PCL-COLL random nanofiber scaffolds illustrated the characteristic peaks of collagen. A prominent amide I band of collagen Type I for C=O stretch, which is the measure of secondary structure of proteins, was obtained at 1723 cm^{-1} in PCL-COLL. Amide II peak for N–H bend coupled with C–N stretch was obtained at 1570 cm^{-1} and amide III region for N–H bend associated with triple helix structure of collagen was obtained between 1200 and 1250 cm^{-1}(Figure 2A) [21]. Similarly characteristic peaks of PCL-PLGA were also observed. FTIR analysis of PCL-PLGA showed absorption at 1723 cm^{-1} corresponding to C=O stretch and C-H bend at 1457 cm^{-1}; COC stretching at 1240 cm^{-1} could be attributed to typical PCL peaks (Figure 2B) [22]. DSC curves of PCL-Collagen and PCL-PLGA revealed melting point of the nanofibrous scaffolds to be at 57.43 °C and 56.81 °C respectively (Figure 2C and 2D). The presence of native functional groups which form the backbone of the polymer reveals that the polymer did not lose its primary structure after electrospinning process.

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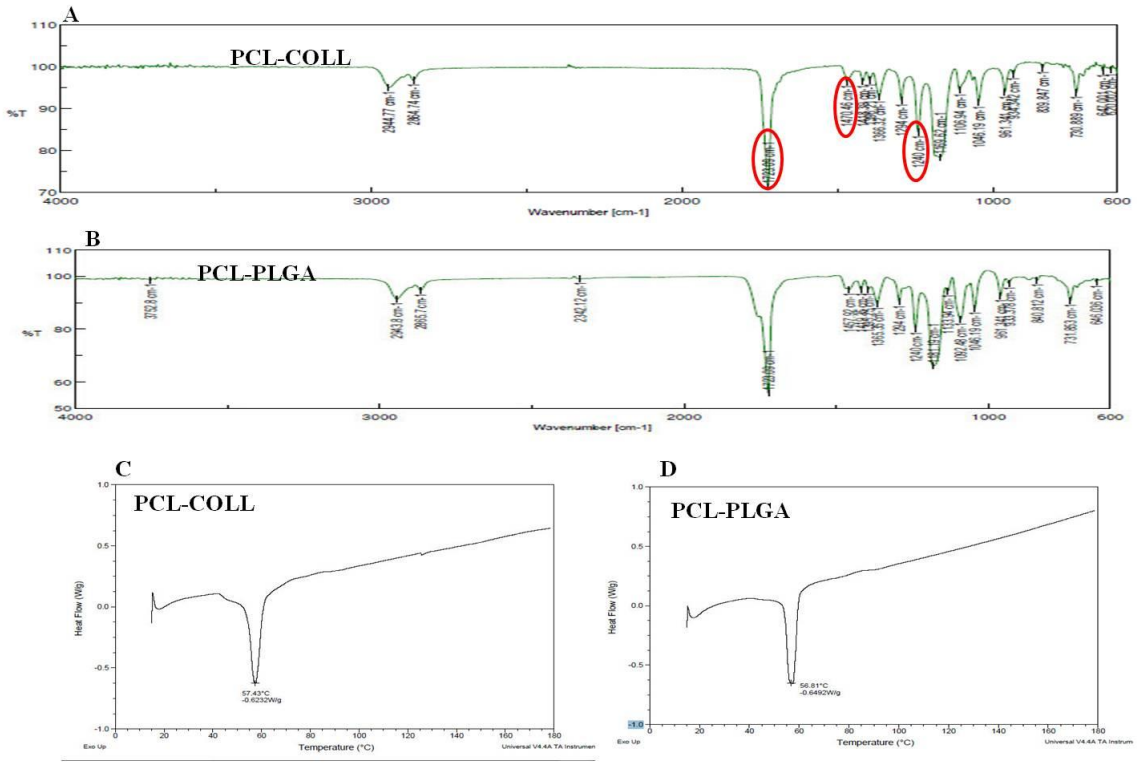

Figure 2: Characterization of nanofibrous scaffolds. A. FTIR spectra of PCL-collagen highlighting (red circles) amide I, II and III peaks of collagen. B. FTIR spectra of PCL-PLGA nanofibrous scaffold. C &D. DSC curves of PCL-collagen and PCL-PLGA.

***In vitro* cytotoxicity of nanofibrous scaffolds**

An ideal scaffold for tissue engineering needs to be biocompatible and function without disrupting the physiological process, mimic the extracellular matrix milieu there by promoting cell migration and proliferation. Cytotoxicity of the nanofibrous scaffolds PCL-COLL and PCL-PLGA was analysed by MTT assay. We analyzed 3D nanofibrous scaffolds for their ability to support cell attachment and cell proliferation. Mouse embryonic fibroblasts (MEF) cultured on the nanofibers and tissue culture plate for 24, 48 and 72 hrs followed by the measurement of cell viability using MTT dye. Dissolved MTT is converted to an insoluble purple formazan by cleavage of tetrazolium ring by mitochondrial dehydrogenase enzyme and the absorbance of the dye measured at 570 nm directly corresponds to cell number. The average total viable cells were precisely calculated and biocompatibility of nanoscaffolds was determined. Cells seeded on the nanofibrous scaffolds showed well arranged and elongated morphology and no signs of cytotoxicity have been observed (Figure 3B). There is no significant difference in cell number when compared to tissue culture plastic control (Figure 3C) after 72 hrs of culture. Both the nanofibrous scaffolds PCL-COLL and PCL-PLGA promoted the growth of MEF in a similar fashion and exerted no decrease in cell viability.

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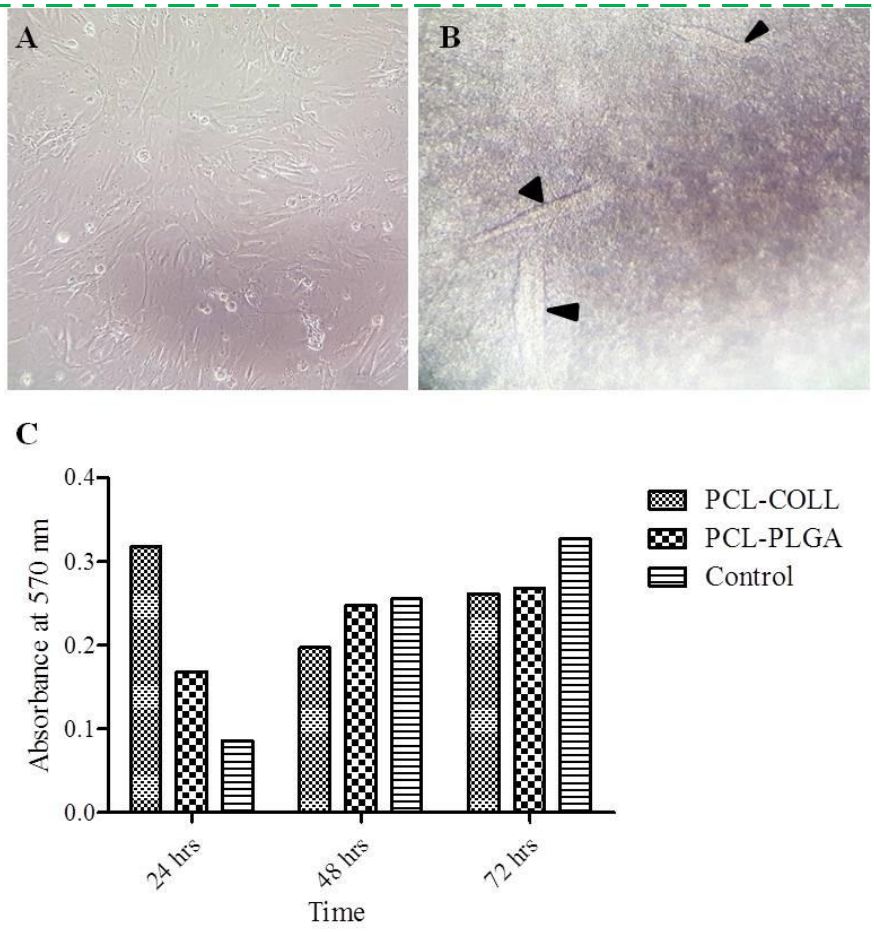

Figure 3: In vitro cytotoxicity assay. A. MEF cultured on tissue culture plate B. MEF cultured on PCL-PLGA nanofibrous scaffold (pointed arrows). C. MTT assay. (n=2)

***In vitro* drug release studies**

The amount of drug encapsulated directly in the nanofibrous scaffolds was measured at different time points spectrophotometrically. The standard curve for the estimation of vancomycin and tetracycline showed linear curve with increasing concentrations of the drug (Figure 4A and 4B). The amount of drug released from the nanofibrous scaffolds was extrapolated from the standard curve. Vancomycin encapsulated in the PCL and PCL-PLGA nanofibrous scaffold showed a burst release of the drug for first 4 hrs after incubation followed by a constant release until 7 days. The encapsulated vancomycin was released from PCL and PCL-PLGA at a mean rate of 147.2 µg/ml and 144.5 µg/ml after 24 hrs of incubation (Figure 4C). Tetracycline encapsulated in PCL and PCL-PLGA scaffolds also showed the burst release of the drug for the first 4 hours after incubation followed by constant release until 7 days. Tetracycline released from PCL and PCL-PLGA scaffolds was found to be 30 µg/ml and 26 µg/ml respectively at 24 hrs after incubation. (Figure 4D). This is a lateral finding in order to protect a wound especially in diabetics or in ischemic tissue conditions where incidence of non-healing is frequent. The biocompatible biodegradable polymeric composite nanofibers regenerate the traumatised tissues and at the same time if it is furnished with medicinal agents or anti infective agents they will enhance healing. This will eventually protect the breached tissue or non healed wound from outside infestation of micro organisms or tissue healing by delivering anti infective drugs as taken in our experiments or tissue mending molecules such as ascorbic acid etc.

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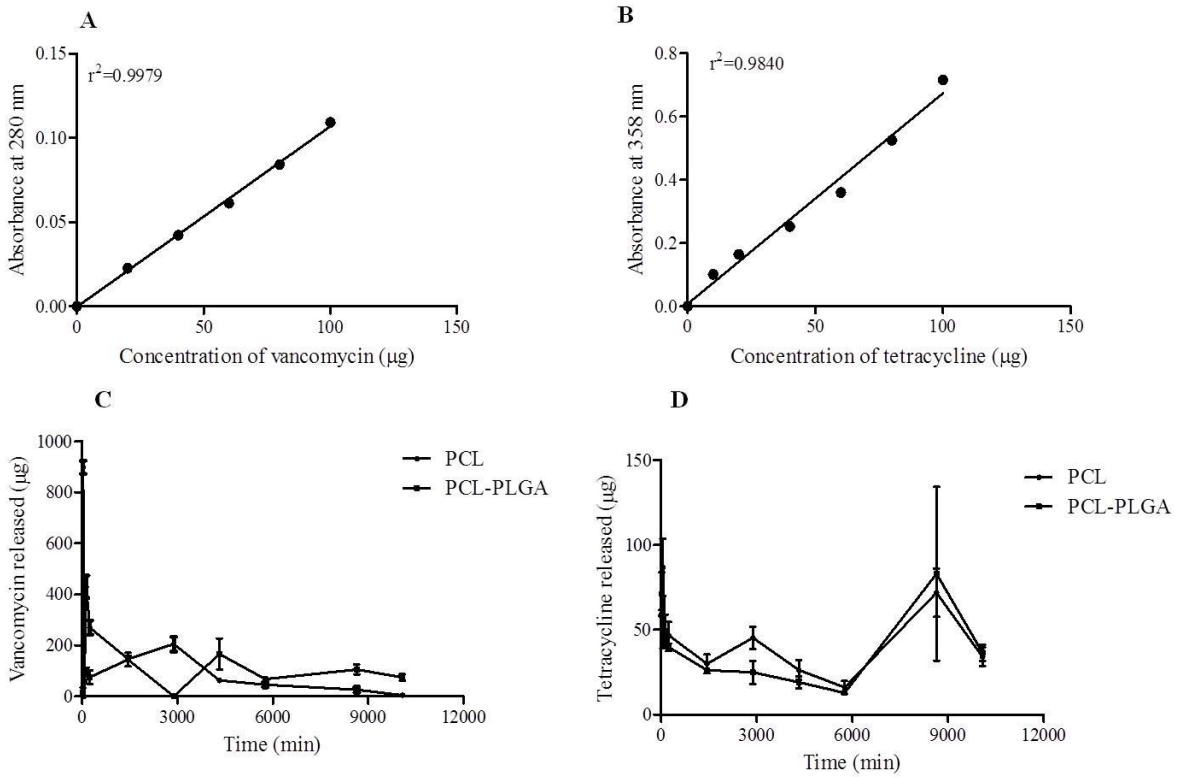

Figure 4: In vitro drug release studies. A & B. Calibration curves for the estimation of vancomycin and tetracycline. C & D. Amount of vancomycin and tetracycline released at different time points encapsulated in PCL and PCL-PLGA nanofibrous scaffolds. (n=3)

Agar diffusion test of drug loaded nanofibrous scaffolds

PCL and PCL-PLGA nanofibrous scaffolds loaded with vancomycin and tetracycline were used to test against *S.aureus.* A clear zone of inhibition around the drug loaded scaffolds was seen after overnight incubation at 37 °C. However these inhibition zones did not appear with the fibers without vancomycin and tetracycline (Figure 5A & 5B). The zone of inhibition for PCL encapsulated vancomycin and tetracycline was found to be 1.2 cm and 1.85 cm respectively. Similarly the zone of inhibition for PCL-PLGA encapsulated vancomycin and tetracycline were found to be 1.45 cm and 1.75 cm respectively (Figure 5C).

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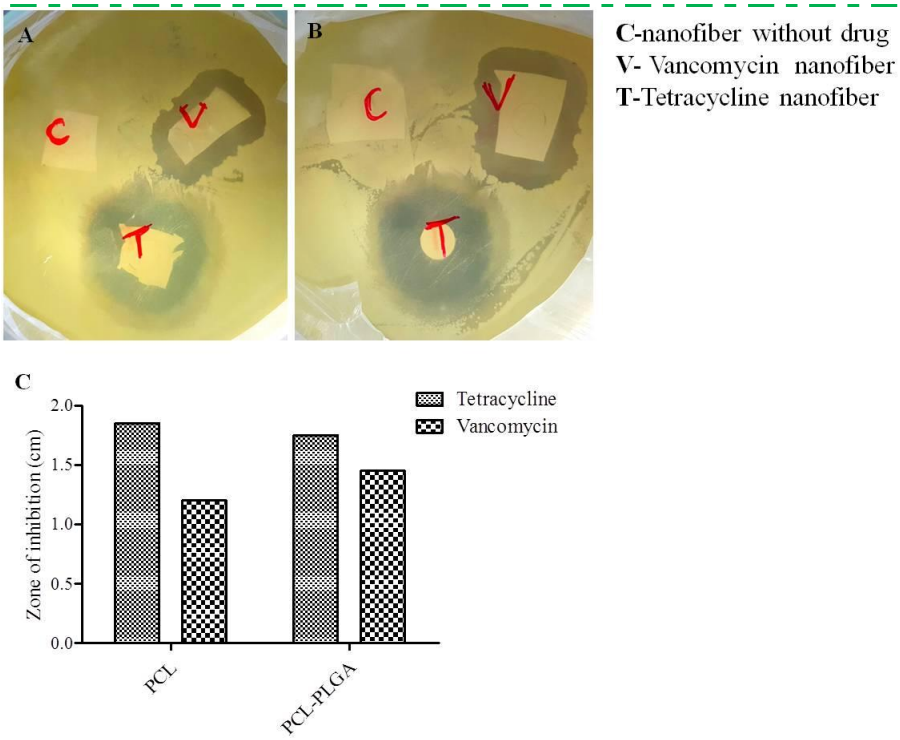

Figure 5: Agar diffusion test. A & B. Agar plates showing the zone of inhibition of drug loaded PCL and PCL-PLGA nanofibrous scaffolds. C. Bar graphs showing the zone of inhibitions. (n=2)

Conclusion

In this study we have successfully fabricated nanofibrous scaffolds from PCL-COLL and PCL-PLGA using electrospinning technique. The characterization of the scaffolds revealed that the native functional groups were conserved in the polymeric composite after electrospinning and the thermal properties of the nanofibrous scaffolds maintained their inherent feature. PCL-COLL and PCL-PLGA nanofibrous scaffolds were found to be biocompatible and broad spectrum antibiotic drugs such as vancomycin and tetracycline were successfully encapsulated. Drug release studies showed the burst release of the antibiotics initially followed by a constant release until 7 days. The inhibitory effect of vancomycin and tetracycline were successfully demonstrated against *S.aureus* by agar diffusion test. Thus nanofibrous scaffolds generated by electrospinning may be considered as a promising tool for drug release studies and multitude of tissue engineering applications.

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Compliance with Ethical Standards

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Disclosure of potential conflicts of interest

All the authors of the manuscript declare no conflict of interest.

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