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Biocompatible nanofibers based on extremophilic bacterial polysaccharide, Mauran from *Halomonas maura*

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ABSTRACT

Extremophilic bacterial polysaccharide based biocompatible nanofibers were produced for the first time *via* electrospinning technique. Mauran (MR), an extremophilic sulfated exopolysaccharide was extracted from moderately halophilic bacterium, *Halomonas maura* and characterized for the application of nanofiber synthesis. Thin-uniform MR nanofibers were produced using homogenous solutions of poly (vinyl alcohol) (PVA) blended with different concentrations of MR. Characterization of complex MR/PVA nanofibers were performed using scanning electron microscope and analyzed for the cytotoxicity using mouse fibroblast cells as well as mesenchymal stem cells. An average of 120 nm sized nanofibers were produced and tested for an enhanced cell growth under *in vitro* conditions in comparison with control. MR and MR/PVA nanofibers were found to be an excellent biomaterial for the migration, proliferation and differentiation of mammalian cells, which was confirmed by cell adhesion studies and confocal microcopy. Interestingly, biological and physicochemical properties of MR hasten the application of MR based nanofibers for various biomedical applications like tissue engineering and drug delivery.

1. Introduction

Nanofibers synthesized from biocompatible and bioactive polymers are of great importance in the new generation biomedicine and nanotechnology. They are well established in the biomedical field for various applications like tissue engineering, wound dressing, drug delivery and enhanced cell adhesion (Vlierberghe, Dubruel, & Schacht, 2011; Dhandayuthapani et al., 2012). Recently optically transparent cellulose nanofibers have opened a wide scope of developing nanofiber matrices for enumerable applications even in the field of microelectronics (Nogi, Iwamoto, Nakagaito, & Yano, 2009). Natural and synthetic polysaccharides are proven to be a good matrix material for generation of excellent tissue engineering scaffolds (Vlierberghe et al., 2011). However, certain polysaccharide raises the question of immunogenicity and hence plant polysaccharides like ulvan and other biocompatible polymers gains more attention (Toskas et al., 2011). Here, we are introducing a biocompatible and biodegradable extremophilic bacterial exopolysaccharides for nanofiber fabrication and tissue engineering studies.

Extremophilic environment is known for its impeccable source of biomaterials like polysaccharides, proteins and other industrially important biomolecules (Nichols, Guezennec, & Bowman, 2005; Poli, Anzelmo, & Nicolaus, 2010). Extremophiles that can with stand a high salt concentration are collectively known as halophiles. *Halomonas maura* is a moderately halophilic bacterium that has the capability of secreting a highly anionic polysaccharide called mauran (MR). The best characteristic of this bacterium is that they can grow well under a salt concentration of 0.5–2.5 M NaCl, although they exist under different hypersaline environments (Arias et al., 2003; Llamas et al., 2006).

MR is an acidic sulfated polysaccharides (SPS) with high uronic acid content. It has been reported that they constitute mannose, galactose, glucose and glucuronic acid as their constituent sugars apart from sulfate and phosphate groups. MR, as a versatile SPS is well known for its classical physical and chemical properties. Viscosity of MR varies according to the salt concentration of the growth medium in which they are produced. Also, they are well known for their pseudoplastic and thixotrophic behavior. This means that viscosity decreases concomitantly with shear rate and then later it regains to normality. Another important property includes the ability to withstand various stress conditions. The viscosity of the solution remains same even under high concentration of salt, sugar, and detergents as well as in extreme pH values and freeze thawing processes. Uptake or binding of heavy metals is another remarkable property of MR which makes it ideal for waste water treatment

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(Arias et al., 2003; Nichols, Lardiere, et al., 2005). MR as a highly charged, gelling polysaccharide can be a future promise for enumerable industrial and biomedical applications both in the form of a novel biomaterial and bioactive agent.

Poly (vinyl alcohol) (PVA) is typically non-toxic, water-soluble, biodegradable and biocompatible synthetic polymer, which is widely used in biomedical applications. PVA has better fiber forming properties and its fibers have been commercialized since 1950s (Zheng, Du, Yu, Huang, & Zhang, 2001). In recent years, much attention has been focused on the biomedical applications of PVA including contact lenses, artificial organs and drug delivery systems (Li, Wang, & Wu, 1998; Razzak et al., 1999). Both PVA and MR were dissolved in water prior to electrospinning and fabrication processes. Electrospinning was carried out in aqueous solution to avoid the trace presence of toxic solvent in the fibers produced.

Electrospinning has recently emerged as a leading technique for the formation of nanofibers because it can produce fibers with diameters ranging from several microns to tens of nanometer depending upon the solution and process parameters (Dhandayuthapani, Yoshida, Maekawa, & Kumar, 2011a, 2011b). The advantage of the electrospinning technique is that, it produces ultra-fine fibers with high surface-to-volume ratio, which have great application potentials in many fields such as protective clothing (Gibson et al., 2002; Gibson, Schreuder-Gibson, & Rivin, 2001), biosensors (Wang et al., 2002), drug delivery system (Kenawy et al., 2002), tissue engineering (Dhandayuthapani et al., 2011a, 2011b), fiber-reinforced composites and template materials for nanotubes (Dhandayuthapani et al., 2011a, 2011b). In addition, low cost apparatus, simple operation, possibility of large-scale production of nanofibers, resulted in the rapid development of electrospinning technique during the recent couple of years. A wide variety of biomaterials have been used in the synthesis of nanofibrous scaffolds, including natural, synthetic, biodegradable, and non-biodegradable polymers (Dhandayuthapani et al., 2011a, 2011b).

In the present work, MR has been isolated from *H. maura* by cold ethanol extraction method. It has been utilized for the fabrication of MR/PVA hybrid biocompatible nanofibrous scaffold by electrospinning. The effects of the MR concentration on PVA, the morphology, diameter of the fibers, the cytocompatibility with mouse fibroblasts (L929) and mesenchymal stem cell (KUSA) as well as the ability of MR/PVA fibers to support cellular adhesion and proliferation were investigated.

2. Experimental

2.1. Materials

2.1.1. Bacterial strain and mammalian cell lines

Moderate halophilic bacteria *H. maura* was purchased (ATCC 7000995) and culture was propagated as per the product information sheet. On solid medium, the colonies were creamy, raised, glistening, circular and entire (O'Donnell & Shukla, 2008). Mesenchymal stem cell line derived from C3H/He mouse (KUSA-A1) and Mouse connective tissue (L929) fibroblast cells were procured from Riken Bio Resource Centre, Japan for conducting biocompatibility and cell culture studies.

2.1.2. Other materials

PVA hydrolyzed 87–89% with a M_w = 31,000–50,000 used for electro spinning and Dulbecco's modified Eagle's medium (DMEM) used for cell culture studies were purchased from Sigma Aldrich. Fetal bovine serum (FBS), penicillin, streptomycin, amphotericin B, alamar blue, 4′,6-diamidino-2-phenylindole (DAPI), and mitotracker green FM were purchased from Gibco, Invitrogen, USA. All other chemicals used were of reagent grade.

2.2. MR production and extraction

H. maura was grown in MY medium as mentioned elsewhere (Arias et al., 2003). Briefly, the growth medium composition: NaCl, 51.3 g; $MgCl_2{\cdot}6H_2O$, 9 g; $MgSO_4{\cdot}7H_2O$, 13 g; $CaCl_2{\cdot}2H_2O$, 0.2 g; KCl, 1.3 g; $NaHCO_3$, 0.05 g; NaBr, 0.15 g; $FeCl_3{\cdot}6H_2O$, traces; glucose, 10 g; yeast extract, 3 g; malt extract, 3 g; proteose peptone, 5 g; trace salt solution, 0.00325 g. Bacto agar (2 g/l) was added for the preparation of solid medium. Medium was prepared and sterilized and inoculated with 1 ml of 48 h culture grown in the same medium (OD_{520} = 2.5) and incubated at 32 °C in a rotary shaker at 110 rpm for 15 days. Bacterial growth and EPS production was monitored in batch cultures of 1000 ml Erlenmeyer flasks with 500 ml of medium in each. At the end of incubation culture was centrifuged (Himac, CF12RX) at 12,000 rpm for 1 h at 4 °C. Supernatant was precipitated with cold ethanol and again ultracentrifuged. Pellet was dissolved in distilled water and dialyzed against distilled water (3–4 exchanges) for 48 h (Snakeskin pleated dialysis tubing, Thermo scientific, 10,000 MWCO). MR after dialysis was subjected to lyophilization and characterization.

2.3. Electron microscopy of the strain

Electron microscopic studies of *H. maura* were performed using transmission electron microscope (TEM) and scanning electron microscope (SEM). Bacterial cells were taken from the mid exponential phase culture of *H. maura* and subjected to ultra thin sectioning and TEM (JEOL, JEM-2200FS) micrographs were taken. SEM images were recorded in JEOL, JSM-7400F, after fixing the cells to a poly-L-lysine coated glass slides as mentioned elsewhere (O'Donnell & Shukla, 2008).

2.4. Characterization of MR/PVA nanofibers

2.4.1. Chemical analysis

Powdered MR was subjected to various chemical characterization processes. Estimation of total carbohydrates and proteins present in MR was performed using colorimetric analysis as described elsewhere (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956; Smith et al., 1985). Carbohydrate estimation was performed using glucose as the standard and bovine serum albumin was used as standard for protein estimation.

2.4.2. X-ray photoelectron spectroscopy (XPS)

XPS spectra were recorded to find out the chemical composition of MR/PVA nanofiber, PVA and MR sample using KRATOS. Analysis was carried out at a basic pressure of 1.7×10^{-8} Torr and the X-ray source used was anode mono-Al with pass energy of 40 (survey scan). XPS spectra for C, N, O, S, and P were obtained.

2.4.3. Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy was performed to characterize the structure of sulfated polysaccharide, MR containing MR/PVA nanofibers, MR and PVA. 2–3 mg of the sample was mixed with 200 mg of dry KBr pellets, ground thoroughly and mixture was pressed into a 16-mm-diameter mold to prepare pellets for FTIR analysis. Infrared spectra were recorded on a (Perkin Elmer US) with a resolution of 4 cm^{-1} in the region of 4000–400 cm^{-1} (Dev et al., 2010; Gomes-Ordonez & Ruperez, 2011).

2.4.4. Solubility tests for MR

Various polar and non polar solvents were tested for the dissolution of MR before carrying out the electrospinning. 1 mg