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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

of powdered MR was mixed with 3 ml of following solvents: water, acetic acid, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), trifluoroethanol (TFE), chloroform and tetrahydrofuran (THF). Each respective mixture was vortexed for 10 min to check the solubility of MR.

2.5. Preparation of MR/PVA solutions for electrospinning

The spinning solutions were prepared from single solvent system. MR was dissolved in 2 ml distilled water at different concentration (6, 15, 20 and 30 mg). The PVA (12 wt%) solution was prepared by dissolving PVA powder in 8 ml of distilled water at ~90 °C under constant stirring for 6 h. When the solution was cooled to room temperature, MR solution was added to PVA solutions, the mixtures were maintained under constant stirring for overnight to acquire the homogenous solution.

2.5.1. Viscosity measurements

In an attempt to correlate the rheological properties of various electrospinning solutions to electrospinnability, the viscosity of PVA (12 wt%), MR (10 mg) and MR/PVA mixture (6, 15, 20 and 30 mg of MR concentrations respectively in 12 wt% PVA solution) in distilled water was measured. The rheological measurements were performed on a Bohlin Gemini 200 HRnano Rheometer (Bohlin Instruments, UK) with a CP 4/40 cone-plate geometry (cone diameter 40 mm, angle 4°). The shear rate was linearly increased from 0.05 to 150 s^{-1}.

2.6. Electrospinning process

To fabricate the ultrafine composite nanofibrous scaffold we have used NANON electrospinning setup (NANON-01A, MECC Co., Ltd., Fukuoka, Japan). For electrospinning, 10 ml of each kind of MR/PVA solution was loaded into a 10 ml glass syringe and injected through an 18G stainless-steel blunt-ended needle. The syringe was then placed in a syringe pump at a flow rate of 1.5 ml h^{-1}. The electric field 2.0 kV cm^{-1} (expressed in terms of voltage/distance) between the collection plate (cathode) and the needle tip (anode) was applied. The collector plate was covered with aluminum foil. The plate was positioned at a distance of 10 cm from the needle to collect the electrospun fibers. All electrospinning processes were performed at ambient temperature.

2.7. Characterization of MR/PVA hybrid nanofibers

2.7.1. SEM analysis

Electrospun MR/PVA nanofibers were dried under vacuum in a desiccator at room temperature and uniformly coated with platinum for its morphological examination. Nanofibers were characterized using JEOL, JSM-7400F, SEM at an accelerating voltage of 5 kV and magnifications from 1000 to 20,000. SEM images recorded were used for the evaluation of the fiber diameter. 10–15 individual fiber diameters were measured and averaged to find the mean diameter value of the resulting nanofiber scaffold.

2.8. In vitro cytotoxic studies

2.8.1. Cell culture

Biocompatibility in terms of proliferation and attachment of mammalian stem cells to electrospun MR/PVA hybrid nanofibrous membranes as scaffolding materials can be analyzed using cell culture studies. Cytotoxicity of the nanofibers was tested using two different cell lineages, like mesenchymal stem cell line derived from C3H/He mouse (KUSA-A1) and Mouse connective tissue (L929) fibroblast cells.

Mesenchymal stem cells (KUSA-A1) were cultured and maintained using T33 tissue culture flasks in complete media consisting of DMEM with 1% L-glutamine supplemented with 15% FBS and 1% antibiotics–antimycotics (final concentration: penicillin 100 μ/ml, streptomycin 100 μ/ml and amphotericin B 0.25 μ/ml). Cells were cultured in a 5% CO_2 incubator at 37 °C and the medium was replaced once in every 2 days.

Mouse connective tissue (L929) fibroblast cells were cultured and maintained using T33 tissue culture flasks in DMEM with 1% L-glutamine, supplemented with 10% FBS and 1% antibiotic–antimycotic formulation (containing penicillin-G-sodium and streptomycin sulfate). The medium was replaced once in every 2 days and the cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.8.2. Alamar blue assay

In vitro cytotoxicity of the MR/PVA nanofibers were studied using alamar blue reduction assay in a 96-well microtiter plate. The method involves the quantification of the metabolically active cells that has the ability to convert resazurin to the fluorescent molecule resorufin. The viability of the cells in turn depends upon the dosage of the test samples that are under studies. Here we use the vacuum dried samples of MR/PVA nanofibers under different ratios in which they were woven. Alamar blue assay was performed as per the standard protocol. In brief, KUSA A1 and L929 cells were seeded separately in the order of $5–8 \times 10^3$ cells/well and incubated at 37 °C in 5% CO_2 atmosphere. After 24 h, medium in the wells were replaced with fresh medium with MR, PVA and MR/PVA nanofibers of 4 different ratios. After addition, cells were incubated for another 24 h under same conditions. At the end of incubation, 10% of alamar blue dye was added to all wells and after 4 h incubation, fluorescence was measured at 580–610 nm, using a multi detection microplate reader (Dainippon Sumitomo Pharma, Powerscan HT). Assay was performed in duplicates and percentage of cell viability was calculated using the following formula:

$$\%\ \text{of cell viability} = \frac{[A]_{\text{test}}}{[A]_{\text{control}}} \times 100$$

where $[A]_{\text{test}}$ is the absorbance of the test sample and $[A]_{\text{control}}$ is the absorbance of control sample.

2.8.3. Crosslinking of nanofibers

MR/PVA nanofibers were cross-linked using glutaraldehyde and trifluoroacetic acid (TFA) vapors to reduce the water solubility during the cell adhesion studies. 3 ml of 50% glutaraldehyde solution and 200 μl of TFA solution were placed separately in petri plates inside a desiccator holding cover glasses with nanofiber scaffolds. Nanofibers were vapor treated for 24 h under vacuum and the fiber morphology was analyzed using SEM.

2.8.4. Evaluation of cell adhesion and proliferation

Cellular adhesion and proliferation with in the MR/PVA scaffolds were evaluated using fluorescence staining and confocal microscopy. Cross-linked MR/PVA scaffolds made of optimized concentration of both MR and PVA solutions were used for cellular adhesion and proliferation studies. MR/PVA scaffolds fabricated on 30 mm × 30 mm cover glass were placed in glass base dish and UV sterilized. DMEM medium was added to the surface of the fiber holding cover glass and subsequently suspensions of L929 cells (approx. 30,000 cells/cm^2) were deposited to it. Inoculated plates were incubated at 37 °C for 24–48 h. At the end of incubation, cells were stained with DAPI nuclear stain and mitotracker green FM, mitochondrial stain for 15 min respectively. Fluorescent stained cells were observed through confocal microscope, Olympus- IX81, inverted microscope installed with

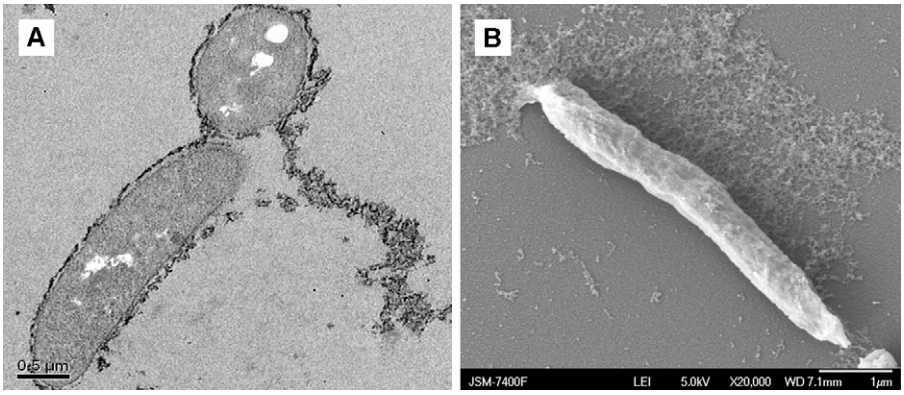

Fig. 1. (A) TEM micrograph of ultra thin section of *H. maura* showing the accumulation and release of mauran surrounding the bacterial cell wall; (B) SEM micrograph of *H. maura* with mauran, adhered to poly-L-lysine coated glass slide.

YOKOGAWA- CSU- XI at 405 nm (DAPI) as well as 488 nm (mitotracker) and fluorescent images of cells adhered to the nanofibers were captured.

3. Results and discussion

3.1. Bacterial studies and MR characterization

Moderately halophilic bacterium, *H. maura* was cultured in MY medium, supplemented with 5% of NaCl and was incubated at 32 °C for 15 days under a constant agitation of 110 rpm. Fig. 1A shows the TEM image recorded of an ultra thin section of *H. maura* with accumulation of mauran surrounding the cell. Granular polysaccharides were attached to the cellwall giving a blanket like appearance. Fig. 1B depicts the SEM image of the mauran accumulated bacteria adhered to poly-L-lysine coated glass slide. Bacterial culture was subjected to cold ethanol extraction after the incubation for 15 days. Supernatant obtained after centrifugation of the culture medium was mixed with double the volume of ethanol and stored at 4 °C overnight. Precipitate formed was removed by centrifugation and dissolved in ultra pure distilled water. Raw MR solution was purified using snake skin dialysis membrane against ultra pure distilled water at 4 °C for 48 h with 2 exchange of the dialysis medium. Purified MR solution was subjected to lyophilization using a freeze dryer (Taitec, VD-550R) and mauran in the powder form was obtained.

Chemical characterization of the purified MR using colorimetric reactions revealed that MR consists of 73 ± 0.02% of total carbohydrates and 2.9 ± 0.5% of protein content. As already reported by Arias et al. (2003), MR is an anionic sulfated polysaccharides with high uronic acid content. MR produced under optimal conditions posses glucose, mannose, galactose and galacturonic acid as four constituent sugar components. It also has a high sulfate content of 6.5% (w/w) which contributes to its polyanionic nature. Furthermore, MR also contains 1.3% of phosphate residues (Arias et al., 2003). The presence of Sulfur and Phosphorus was confirmed by elemental analysis of MR using XPS. Fig. 2 shows the characteristic ESCA peaks of elements in MR. The peak showing at 168 eV depicts sulfate peak of mauran. Polysaccharide structure and the presence of sulfate moiety were estimated using FTIR spectra of MR in Fig. 3. As common to all polysaccharides two characteristic peaks appeared at 3432 cm^{-1} and 2932 cm^{-1} assigned to O—H and C—H stretching vibrations respectively. Similarly, strong absorption bands at 1131 cm^{-1} and 1057 cm^{-1} may be assigned to C—O and C—C stretching in the pyranoid ring. In addition, the band at 979 cm^{-1} can be due to C—O—C stretching of glycosidic linkages. Two strong peaks at 1739 cm^{-1} and 1650 cm^{-1} can be assigned to carbonyl groups in two respective forms, carboxylic ester (C=O) form and carboxylate anionic (COO^-) form. These carboxylic stretching vibrations can be from the uronic acid residues in MR. Spectral vibration at 1419 cm^{-1} may be assigned to C—OH deformation vibration of carboxylate symmetric stretching. The

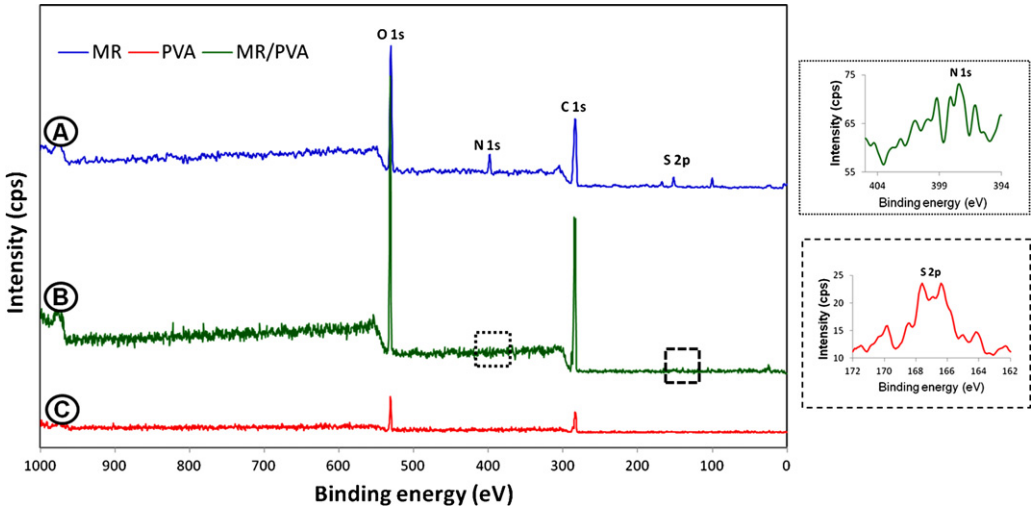

Fig. 2. XPS elemental analysis spectra for: (A) mauran (MR); (B) MR/PVA; (C) PVA. Two boxes inset corresponds to N 1s and S 2p peaks of MR/PVA spectrum that has been shown separately outside.

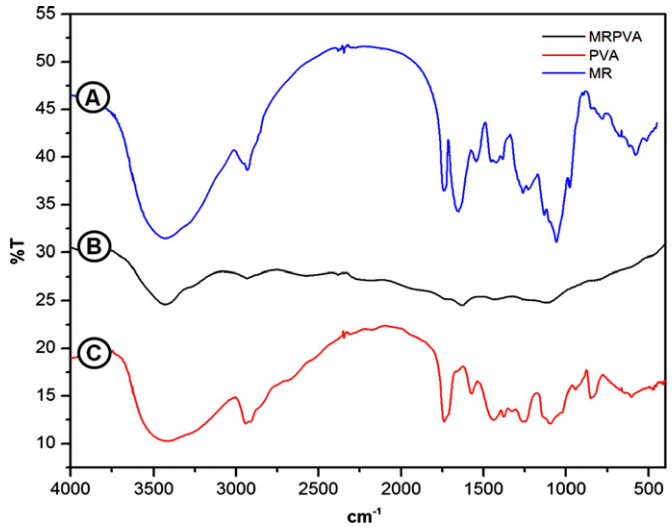

Fig. 3. FTIR analysis spectra for: (A) mauran (MR); (B) MR/PVA; (C) PVA.

sharp peak at 1261 cm^{-1} and its shoulder vibration at 1225 cm^{-1} confirm the presence of sulfate ester groups (S=O), which is a characteristic component of MR (Hosoyo, Balzarini, Shigeta, & Clercq, 1991; Liu, Jiao, Wang, Zhou, & Zhang, 2008).

Results of solubility tests for MR revealed that MR is not soluble in any of the organic solvents used. It was found that MR is highly soluble in water and partially soluble in 100% acetic acid. Since it was partially soluble in 100% acetic acid, various other concentrations were also tested, but it showed only a similar dissolution pattern. This confirms the hydrophilic nature of MR. It has been reported that most of the water soluble polysaccharides are highly bioactive in nature (Costa et al., 2010; Mestechkina & Shcherbukhin, 2010; Nie, Shi, Ding, & Tao, 2006; Wijesekara, Pangestuti, & Kim, 2011). Also it is proven to be good therapeutic agent as it extended anti-proliferative and immunomodulating effects in human cancer studies. MR nanoparticles were synthesized in combination with chitosan for sustained drug delivery, cancer chemotherapy and *in vitro* cellular imaging *via* fluorescent tagging (Llamas et al., 2006; Raveendran, Poulose, Yoshida, Maekawa, Kumar, 2011, http://dx.doi.org/10.1016/j.bbr.2011.03.031). Thus we can safely conclude on the basis of the chemical structure, solubility and biological properties that MR is an ideal bioactive agent.

3.2. Properties of MR/PVA solution and electrospinning

3.2.1. Viscosity

Fig. 4 shows the rheological behavior of PVA, MR and MR/PVA solutions with different concentrations of MR (6, 15 20 and 30 mg respectively). Shear viscosity has been plotted against shear rate. The viscosity is nearly stable in the range of high shear rates. At low shear rates, molecules with preferred conformations that are long and thin are not oriented very well in the direction of the flow and have large effective cross-sections, leading to a relatively high viscosity. Viscosity was altered in the presence of high quantities of MR in PVA solution. The viscosity of 10 mg/ml concentration of MR solution alone showed a very low viscosity (0.38 Pa s). In parallel, at 6 mg MR concentration in PVA solution, the viscosity was slightly altered. At the same time, MR/PVA solutions show an interesting rheological property, which increases from 0.8 to 3.9 Pa s with the increasing concentrations of the MR. The highest concentration of MR in PVA solution tested, which is 30 mg, was having a higher viscosity value of 3.9 Pa s. Our result shows that MR is highly compatible with PVA and demonstrates its stability under different stress conditions. Viscosity permits MR/PVA based solutions to electrospin and allows nanofibers of progressively uniform morphology.

3.3. Characterization of MR/PVA nanofibers

3.3.1. SEM analysis

Fig. 5 shows the SEM images of the electrospun PVA nanofibers. The solution concentration of 12% by weight was chosen to fabricate nonwovens of randomly arranged PVA fibers with nanometer scale diameters. From the image analysis, they have an average diameter of 100 nm (diameters ranged from 60 to 130 nm). We observed that ultrafine PVA nanofibers electrospun from PVA water solution were fused at their contact sites, which formed three dimensional fiber mats (Fig. 5B) due to the slow evaporation of water.

SEM micrographs of MR/PVA blended nanofibers prepared under various concentrations of MR (6, 15, 20 and 30 mg) are shown in Fig. 6. Identical morphological features of individual fibers with large aspect ratio can be easily seen. Nanofibers fabricated at 6 mg concentration were having a uniform diameter with various interconnected pores. Here, the mean diameter was found to be 110 nm (Fig. 6B). While increasing the concentration from 6 to 30 mg, we found that the nanofibers were showing smooth and uniform morphology similar to those at 6 mg concentration. However, the mean diameter was increased to 130 nm in size (Fig. 6H). The size of the electrospun MR/PVA nanofibers were measured in the range of 70–160 nm in diameter with a relatively broader size distribution than the pure PVA nanofibers. We have observed that the large population of smaller fiber and uniform nanofiber morphology were fully retained and had any significant difference in the fiber morphology on varying MR concentrations. This demonstrates the homogeneous structure of the scaffolds. Furthermore, the studies on the electrospun MR/PVA system shows that it can be ideally used for the formation of continuous and uniform nanofibers. We recommend 15 and 20 mg MR as the preferred concentration for preparing the spinning solution with 12 wt% of PVA. Almost all the fibers produced under these two concentrations were exhibiting a smooth morphology and uniform diameter size distribution (Fig. 6D and F).

Fig. 2 compares the XPS spectra of MR, PVA and MR/PVA scaffolds. The presence of Sulfur and Nitrogen in the synthesized MR/PVA nanofiber spectra confirms the presence of MR in the MR/PVA scaffolds. However, the intensity of both the peaks were comparatively lesser (Fig. 2 inset images) compared to that of MR alone. This is due to the decreased concentration of MR with that of PVA in the MR/PVA mixture. Fig. 3B and C shows the FTIR spectra of MR/PVA and PVA nanofibers respectively. The PVA nanofiber exhibited stretching vibration band of hydrogen bonded alcohol (O—H) at about 3435 cm^{-1}. An overlapped and weak band at 2927 cm^{-1} is from —CH stretch. The characteristic peak at 1110 cm^{-1} is assigned to the asymmetric stretching of C—O of PVA. The peak at 1734 cm^{-1} (C=O) and 1635 cm^{-1} (COO^-) are observed in the spectra of MR/PVA nanofiber, indicating the presence of MR in the fibrous scaffold. However, the FTIR spectrum conveys the existence of relevant functional groups of both PVA and MR in the nanofibers synthesized.

3.4. Cytotoxic studies

Cytotoxicity of the prepared MR/PVA scaffold was analyzed using alamar blue assay. The assay is based on the principle that the growth of cells results into metabolic activities which results into chemical reduction of alamar blue dye. Fig. 7A and B shows the cell viability graph of the KUSA and L929 cell lines on the MR/PVA nanofibrous scaffold, PVA nanofiber, raw MR and control after different days of intervals, 1, 2 and 3 days, respectively. At day 1 the viability of cells on the scaffold exhibited the similar

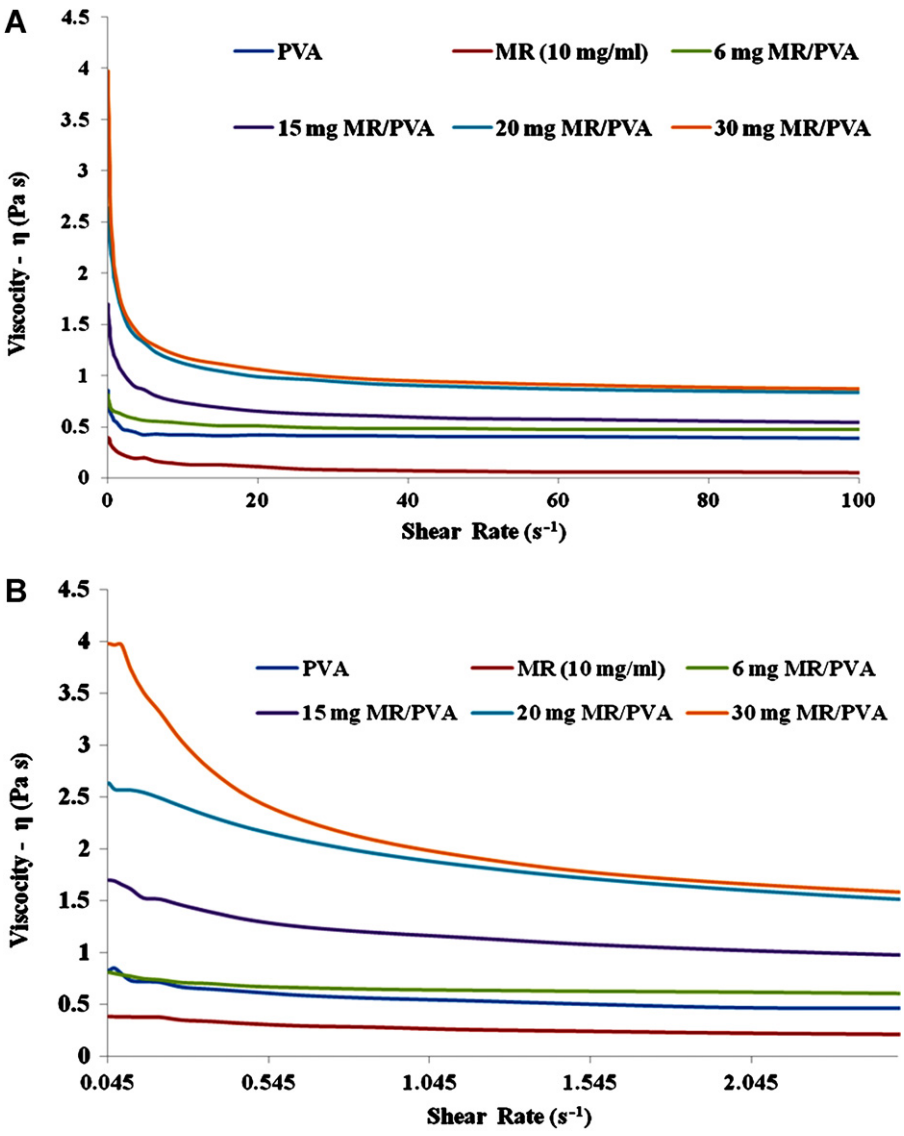

Fig. 4. Shear viscosity and shear rate relationship for the PVA, MR, MR/PVA (6 mg, 10 mg, 15 mg, 20 mg, and 30 mg) solutions.

tendency. A time depended increase in cell viability was observed for MR/PVA scaffold, when it was analyzed for three different time points. After 2 days, the cells did proliferate and their number increased over time, and we have observed that MR/PVA nanofibers had more cells than PVA nanofiber, raw MR and control, which clearly points toward the non toxic behavior of MR/PVA scaffold. Since the increase in cell viability is the direct measure of number of living cells, our results clearly suggest that cell proliferation is taking place at steady rate. The percentage of cell viability for MR/PVA scaffolds was significantly higher compared to control at day 2 and

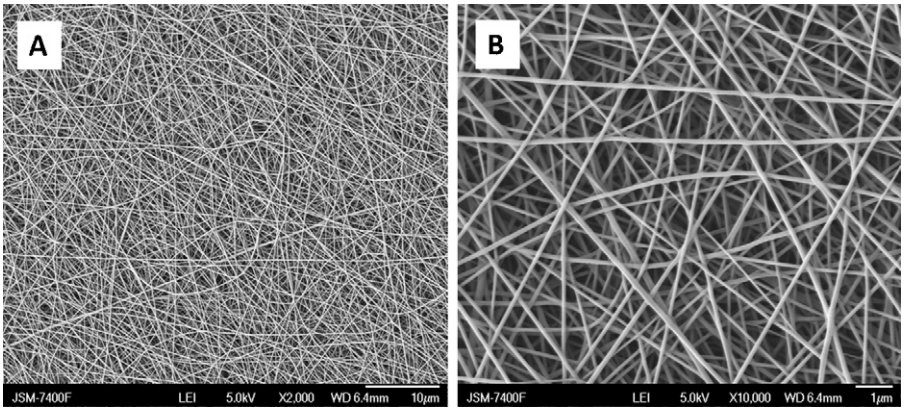

Fig. 5. SEM micrographs of electrospun PVA nanofibers at magnifications: (A) 2000× and (B) 10,000×.

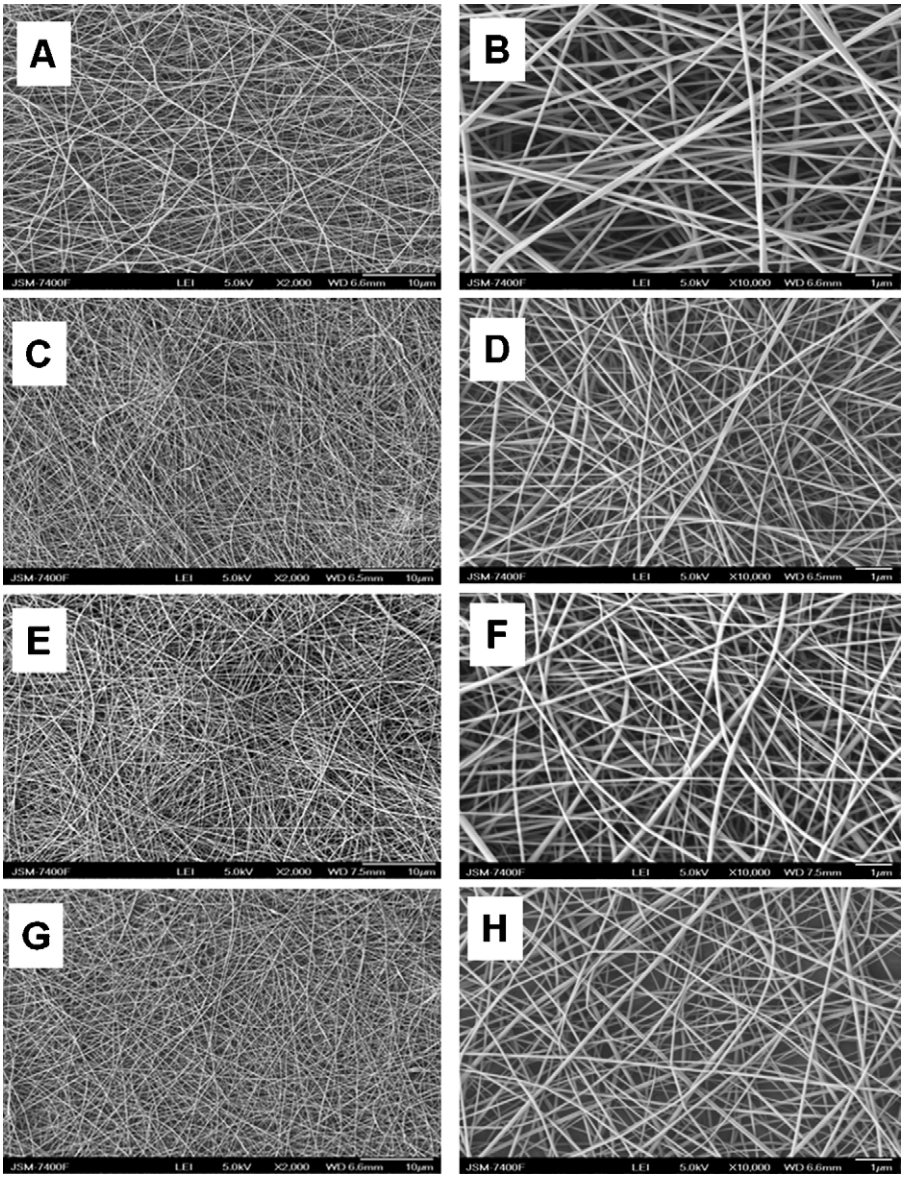

Fig. 6. SEM micrographs of electrospun MR/PVA nanofibers with various concentrations of MR blended with PVA; at magnifications 2000× and 10,000× (A and B) 6 mg MR/PVA; (C and D) 15 mg MR/PVA; (E and F) 20 mg MR/PVA; (G and H) 30 mg MR/PVA.

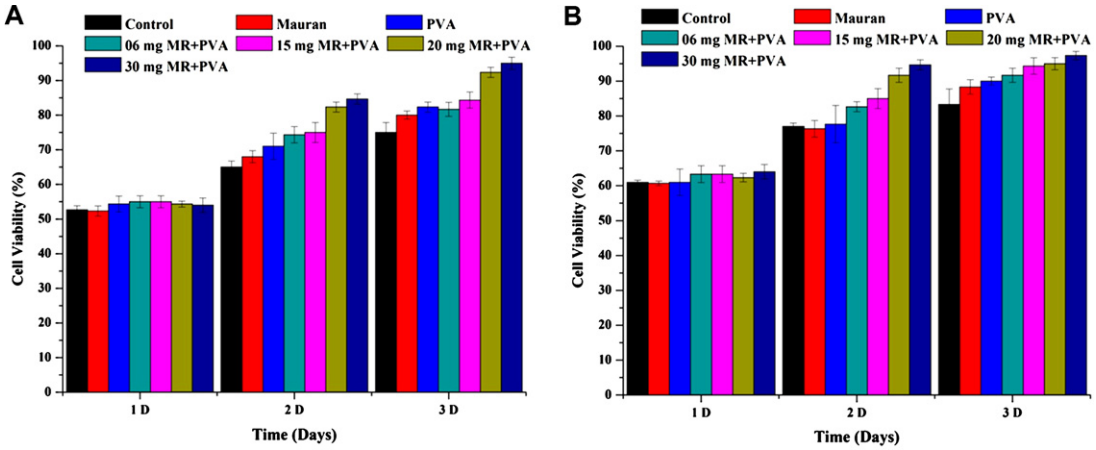

Fig. 7. Cell viability on hybrid nanofiber: (A) mesenchymal stem cells (KUSA-A1) cultured on control (TCPS), PVA nanofiber, mauran, MR/PVA (6, 15, 20 and 30 mg) hybrid nanofibrous scaffold for 1–3 days; (B) fibroblasts (L929) cultured on control (TCPS), PVA nanofiber, mauran, MR/PVA (6, 15, 20 and 30 mg) hybrid nanofibrous scaffold for 1–3 days.

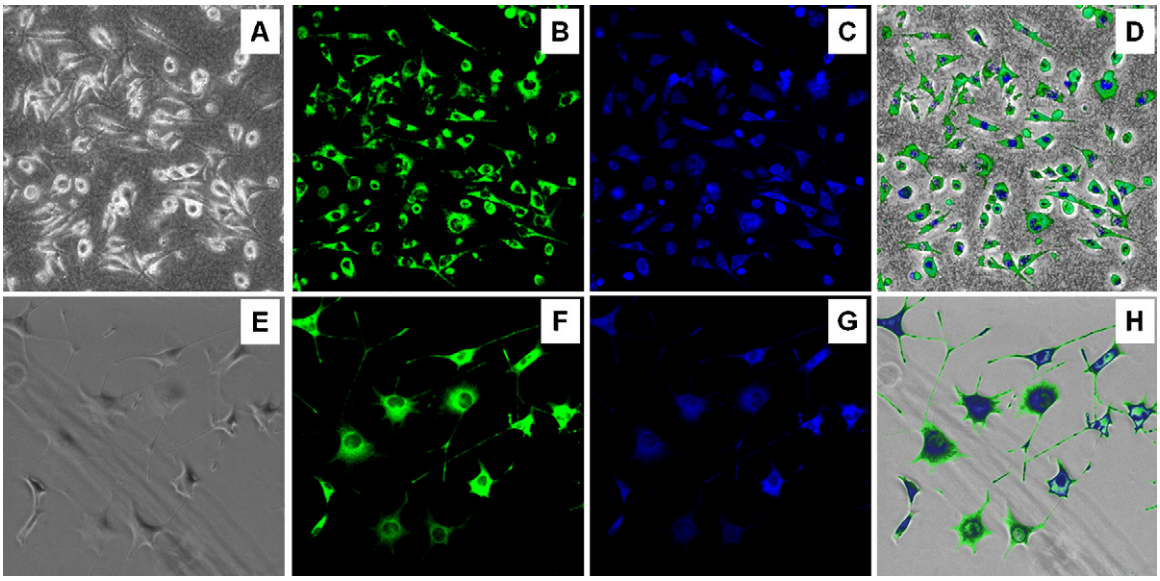

Fig. 8. Confocal microscopy images for cellular adhesion studies after florescent staining: (A) bright field image showing L929 cells attached and proliferating on MR/PVA nanofibers; (B) mitotracker green stained image; (C) DAPI stained image; (D) merged image of A, B and C; (A–D: magnification, 20×); (E) bright field image showing the spread of L929 cells; (F) mitotracker green stained image; (G) DAPI stained image; (H) merged image of E, F and G; (E–H: magnification, 60×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Owing to the rapid proliferation rate of L929 cells, the cell viability data showed is much higher compared to that of KUSA cells. Thus it is proved that MR and MR/PVA nanofibers are good biocompatible material for the migration, proliferation and differentiation of mesenchymal stem cells and fibroblast cells as compared to that of control. From our studies it has been demonstrated that it can be applied for various tissue engineering applications after detailed evaluation of its *in vivo* properties.

3.5. Cellular adhesion and proliferation studies

Nanofibers crosslinked with glutaraldehyde and TFA showed comparatively lesser water solubility. Also, the SEM observation of crosslinked MR/PVA nanofibers showed no significant change in the fiber morphology after crosslinking. Cell adhesion and proliferation studies performed using L929 cells by fluorescent staining showed the ability of MR/PVA nanofibers to provide effective focal adhesion for cell attachment and proliferation. Fig. 8A shows the bright field image of MR/PVA nanofibers with L929 cells attached to it. Fig. 8B and C depicts the corresponding fluorescent images showing mitotracker stained skeleton and DAPI stained nucleus respectively. Fig. 8D shows the merged image of bright field and fluorescent images. Fig. 8E shows the bright field image of L929 cells spreading over and anchoring MR/PVA nanofibers from a distant area after 48 h of incubation. Fig. 8F–H shows the corresponding fluorescent images and merged image respectively. Observed cellular adhesion is likely due to the polyanionic nature of the constituent MR that increases the negative charge accumulation on the scaffold surface that is more conducive to protein adsorption leading to an enhancement of cellular attachment (Arias et al., 2003; Berry et al., 2011). This demonstrates the better ability of MR/PVA fibers to support migration and proliferation effect of cells even from a distant surrounding area. Hence it can be confirmed from the fluorescent staining and confocal studies that the newly synthesized MR/PVA nanofibers are highly biocompatible and effectively facilitates cellular adhesion, migration and proliferation.

4. Conclusion

The elctrospun nanofibrous membrane of MR/PVA blends was fabricated. Nanofibers have been successfully prepared by electrospining of mixed aqueous solution of MR and PVA. These novel electropsun matrices have the potential to be used as a support material for various applications in the biomedical industry. Thin-uniform nanofibers were produced using electrospinning under ambient conditions with four different concentrations of MR with PVA. A broad range of diameter distribution was observed with respect to various concentrations of MR. The most significant acheivement was the smooth and the uniformity of the nanofibers irrespective of different concentrations used. MR/PVA blended nanofiber has a least diameter measured down to 70 nm with less interconnections and nonwoven appearance. Rheological properties studied for MR/PVA solutions revealed that the viscosity of the spinnable solution was directly proportional to the concentration of MR. It also ensured that the rheological properties remained unchanged even under various stress conditions. The thixotrophic and viscoelastic properties of MR are highly promising and hence currently being tested for its various mechanical properties. Biocompatibility of MR/PVA nanofibers against mesenchymal stem cells and connective tissue ensures the futuristic application of MR nanofibers in tissue engineering field. The ability of MR containing nanofibers to boost the cellular adhesion, migration, proliferation and differentiation is a remarkable finding in the bacterial polysaccharide research proving its non-antigenecity toward living tissue. An enhancement of cell proliferation could facilitate the use of MR/PVA nanofibers in wound dressing and drug delivery. Various properties of MR in the field of food, pharmaceutics and industry can be positively exploited by introducing as a novel nanofiber for various applications. Furthermore, the immunomodulating and antiproliferative effects of MR on cancer cells extends a promising characteristic that could unveil the potentiality of the biocompatible biomaterial from rich extreme environment.

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References

Arias, S., Moral, A. D., Ferrer, M. R., Tallon, R., Quesada, E., & Bejar, V. (2003). Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. *Extremophiles*, *7*, 319–326.

Berry, S. M., Warren, S. P., Hilgart, D. A., Schworer, A. T., Pabba, S., Gobin, A. S., et al. (2011). Endothelial cell scaffolds generated by 3D direct writing of biodegradable polymer microfibers. *Biomaterials*, *32*, 1872–1879.

Costa, L. S., Fidelis, G. P., Cordeiro, S. L., Oliveira, R. M., Sabry, D. A., Camara, R. B. G., et al. (2010). Biological activites of sulfated polysaccharides from tropical seaweeds. *Biomedicine and Pharmacotherapy*, *64*, 21–28.

Dev, A., Binulal, N. S., Anitha, A., Nair, S. V., Furuike, T., Tamura, H., et al. (2010). Preparation of poly(lactic acid)/CH nanoparticles for anti-HIV drug delivery applications. *Carbohydrate Polymers*, *80*, 833–838.

Dhandayuthapani, B., Saino, H. V., Aswathy, R. G., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2012). Evaluation of antithrombogenicity and hydrophilicity on Zein-SWCNT electrospun fibrous nanocomposite scaffolds. *International Journal of Biomaterials*, http://dx.doi.org/10.1155/2012/345029

Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011a). Fabrication and characterization of nanofibrous scaffold developed by electrospinning. *Materials Research*, *14*(3), 317–325.

Dhandayuthapani, B., Yoshida, Y., Maekawa, T., & Kumar, D. S. (2011b). Polymeric scaffolds in tissue engineering application – A review. *International Journal of Polymer Science*, http://dx.doi.org/10.1155/2011/290602

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*(3), 350–356.

Gibson, H. S., Gibson, P., Senecal, K., Sennett, M., Walker, J., Yeomans, W., et al. (2002). Protective textile materials based on electrospun nanofibers. *Journal of Advanced Materials*, *34*, 44–55.

Gibson, P., Schreuder-Gibson, H., & Rivin, D. (2001). Transport properties of porous membranes based on electrospun nanofibers. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, *187–188*, 469–481.

Gomes-Ordonez, E., & Ruperez, P. (2011). FTIR–ATR spectroscopy as a tool for polysaccharide identification in edible brown and red seaweeds. *Food Hydrocolloids*, *25*, 1514–1520.

Hosoyo, M., Balzarini, J., Shigeta, S., & Clercq, E. D. (1991). Differential inhibitory effects of sulfated polysaccharides and polymers on the replication of various myxoviruses and retroviruses, depending on the composition of the target amino acid sequences of the viral envelope glycoproteins. *Antimicrobial Agents and Chemotherapy*, *35*, 2515–2520.

Kenawy, E. R., Bowlin, G. L., Mansfield, K., Layman, J., Simpson, D. G., Sanders, E. H., et al. (2002). Release of tetracycline hydrochloride from electrospun poly(ethylene-co-vinylacetate), poly(lactic acid), and a blend. *Journal of Controlled Release*, *81*, 57–64.

Li, J. K., Wang, N., & Wu, X. S. (1998). Poly(vinyl alcohol) nanoparticles prepared by freezing–thawing process for protein/peptide drug delivery. *Journal of Controlled Release*, *56*, 117–126.

Liu, Z., Jiao, Y., Wang, Y., Zhou, C., & Zhang, Z. (2008). Polysaccharides-based nanoparticles as drug delivery systems. *Advanced Drug Delivery Reviews*, *60*, 1650–1662.

Llamas, I., Moral, A. D., Martinez-Checa, F., Arco, Y., Arias, S., & Quesada. (2006). *Halomonas maura* is a physiologically versatile bacterium of both ecological and biotechnological interest. *Antonie Van Leeuwenhoek*, *89*, 395–403.

Mestechkina, N. M., & Shcherbukhin. (2010). Sulfated polysaccharides and their anticoagulant activity: A review. *Applied Biochemistry and Microbiology*, *46*(3), 291–298.

Nichols, C. A. M., Guezennec, J., & Bowman, J. P. (2005). Bacterial exopolysaccharides from extreme marine environments with special consideration of the southern ocean, sea ice, and deep sea hydrothermal vents: A review. *Marine Biotechnology*, *7*, 253–271.

Nichols, C. M., Lardiere, S. G., Bowman, J. P., Nichols, P. D., Gibson, J. A. E., & Guezennec, J. (2005). Chemical characterization of exopolysaccharides from antarctic marine bacteria. *Microbial Ecology*, *49*, 578–589.

Nie, X., Shi, B., Ding, Y., & Tao, W. (2006). Preparation of a chemically sulfated polysaccharide derived from *Grifola frondosa* and its potential biological activities. *International Journal of Biological Macromolecules*, *39*, 228–233.

Nogi, M., Iwamoto, S., Nakagaito, A. N., & Yano, H. (2009). Optically transparent nanofiber paper. *Advanced Materials*, *21*(16), 1595–1598.

O'Donnell, C. D., & Shukla, D. (2008). The importance of heparan sulfate in herpes infection. *Virologicca Sinica*, *23*(6), 383–393.

Poli, A., Anzelmo, G., & Nicolaus, B. (2010). Bacterail exopolysaccharides from extreme marine habitats: Production, characterization and biological activities. *Marine Drugs*, *8*, 1779–1802.

Raveendran, S., Poulose, A. C., Yoshida, Y., Maekawa, T., & Kumar, S. (2011). Bacterial exopolysaccharide based nanoparticles for sustained drug delivery, cancer chemotherapy and bioimaging. *Carbohydrate Polymers*, http://dx.doi.org/10.1016/j.bbr.2011.03.031

Razzak, M. T., Zainuddin, Erizal, Dewi, S. P., Lely, H., Taty, E., et al. (1999). The characterization of dressing component materials and radiation formation of PVA–PVP hydrogel. *Radiation Physics and Chemistry*, *55*, 153–165.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., et al. (1985). Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, *150*(1), 76–85.

Toskas, G., Hund, R., Laourine, E., Cherif, C., Smyrniotopoulos, V., & Roussis, V. (2011). Nanofibers based on polysaccharides from the green seaweed *Ulva rigida*. *Carbohydrate Polymers*, *84*, 1093–1102.

Vlierberghe, S. V., Dubruel, P., & Schacht, E. (2011). Biopolymer-based hydrogels as scaffolds for tissue engineering applications: A review. *Biomacromolecules*, *12*, 1387–1408.

Wang, X. Y., Drew, C., Lee, S. H., Senecal, K. J., Kumar, J., & Samuelson, L. A. (2002). Electrospun nanofibrous membranes for highly sensitive optical sensors. *Nano Letters*, *2*, 1273–1275.

Wijesekara, I., Pangestuti, R., & Kim, S. (2011). Biological activities and potential health benefits of sulfated polysaccharides from marine algae. *Carbohydrate Polymers*, *84*, 14–21.

Zheng, H., Du, Y. M., Yu, J. H., Huang, R. H., & Zhang, L. N. (2001). Preparation and characterization of chitosan/poly(vinyl alcohol) blend fibers. *Journal of Applied Polymer Science*, *80*(13), 2558–2565.