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Modified dextran cross-linked electrospun gelatin nanofibres for biomedical applications

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ABSTRACT

Electrospun gelatin nanofibres attract attention of bioengineering arena because of its excellent biocompatibility and structural resemblance with native extracellular matrix. In this study, we have developed gelatin nanofibres using an innovative cross-linking approach to minimize cytotoxic effects. Gelatin was dissolved in water:acetic acid (8:2, v/v) solution and electrospun to form nanofibres with diameter in the range of 156 ± 30 nm. The nanofibres were cross-linked with a modified polysaccharide, namely, dextran aldehyde (DA). Cross-linking with DA could be achieved without compromising the fibrous architecture. DA cross-linked gelatin nanofibres maintained the fibrous morphology in aqueous medium. These mats exhibit improved mechanical properties and gradual degradation behaviour. The nanofibres were evaluated for cytotoxicity, cell adhesion, viability, morphology and proliferation using L-929 fibroblast cells. The results confirmed that DA cross-linked mats were non cytotoxic towards L-929 cells with good cell adhesion, spreading and proliferation.

1. Introduction

Gelatin is a bio polymer derived by partial denaturation of collagen, a major structural protein present in the human body. Gelatin does not show antigenicity and has high haemostatic properties compared to its precursor collagen. Moreover, gelatin is biodegradable, biocompatible and displays many integrin binding sites for cell adhesion and differentiation (Kuijpers et al., 2000; Lu, Zhu, Zhang, & Sun, 2011). Electrospun gelatin nanofibres are found to be versatile candidates for tissue engineering scaffolds and drug delivery matrices. Gelatin nanofibres (GNFs) when in contact with water, loses the fibrous morphology due to its high hydrophilicity and solubility. Hence, a cross-linking treatment is necessary to improve the water resistant ability as well as mechanical performance to make them suitable for biomedical applications.

Bifunctional cross-linking agents such as glutaraldehyde (Bigi, Cojazzi, Panzavolta, Rubini, & Roveri, 2001; Zhang, Venugopal, Huang, Lim, & Ramakrishna, 2006), hexamethylenediisocyanate (Bertoldo, Bronco, Gragnoli, & Ciardelli, 2007; Li et al., 2005), carbodiimide (Marois et al., 1995) and acyl azides (Kuijpers et al., 2000) are commonly used for cross-linking of gelatin to attain water resistant ability. Cross-linking agents that are incorporated into the gelatin matrix such as glutaraldehyde and isocyanates are proven to be toxic and are susceptible to leach out into the body during matrix biodegradation (Balakrishnan & Jayakrishnan, 2005). Therefore, researchers are interested in developing gelatin based scaffold systems devoid of these types of cross-linking agents. External cross-linking agents were avoided in gelatin hydrogels by using modified polysaccharides as cross-linkers. Cross-linking of gelatin using partially oxidized polysaccharides such as dextran, alginic acid, chondrotin sulphate, and carboxymethyl cellulose have been reported in literature for development of hydrogel systems (Balakrishnan, Joshi, & Banerjee, 2013; Balakrishnan, Mohanty, Umashankar, & Jayakrishnan, 2005; Boanini, Rubini, Panzavolta, & Bigi, 2010; Dawlee, Sugandhi, Balakrishnan, Labarre, & Jayakrishnan, 2005; Schacht, Bogdanov, Bulcke, & De Rooze, 1997). These gels were designed to be used as wound dressings, tissue adhesives and scaffolds for tissue engineering. Biocompatibility of the polysaccharide aldehyde cross-linked gelatin hydrogels were evaluated *in vitro* and *in vivo* and was ranked as acceptable. This was regarded as an excellent alternative cross-linking approach for protein moieties to replace toxic cross-linking agents and being used for development of biocompatible protein based hydrogels. Successful reports of injectable hydrogel based on gelatin and

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oxidized alginate are available (Balakrishnan et al., 2005). Rapid cross-linking occurred between amino groups of gelatin and aldehyde groups of oxidized alginate leading to hydrogel formation at appropriate pH in aqueous medium. Similar methodologies have been adopted for the cross-linking of matrices based on other proteins and polysaccharides also (Dash, Foston, & Ragauskas, 2013; Hu et al., 2014; Nair, Remya, Remya, & Nair, 2011; Xu, Li, Yu, Gu, & Zhang, 2012).

In order to maintain the fibrous morphology when in contact with water, GNFs are subjected to cross-linking treatment with various cross-linking agents, the most effective one being glutaraldehyde (Zhang et al., 2006). Cross-linking is achieved either by dipping the nanofibres in glutaraldehyde solution or by exposure to vapours. Cross-linking can also be achieved by carbodiimide chemistry. In this case, nanofibres are dipped in ethanolic solution of 1-ethyl, 3,3-dimethyl aminopropylcarbodiimide in presence of N-hydroxysuccinimide (Li, Guo, Wei, MacDiarmid, & Lelkes, 2006). These materials either released into the body due to degradation or remaining unreacted in the nanostructures can cause toxicity (Hao et al., 2011; Panzavolta et al., 2011; Zhang et al., 2006). Recently, Panzavolta et al. (2011) have shown that genipin, a naturally occurring material can be an alternative cross-linking agent for GNFs. Cross-linking of the nanofibres is achieved by dipping the nanofibrous mat in ethanolic solution of genipin. Genipin is found to be a better cross-linking agent compared to others in many aspects, but the high cost of genipin can limit the applicability. Cross-linking of GNFs with polysaccharide is another possibility as in the case of gelatin hydrogels. However, polysaccharides are soluble only in aqueous medium where the GNFs will dissolve resulting in the complete destruction of the fibrous morphology. Thus it is necessary to use a suitable cross-linking medium in which polysaccharide is soluble and GNFs are unaffected. Cross-linking of GNFs by this approach has not been described so far.

Here, we report on the feasibility of utilizing modified polysaccharide, namely, dextran aldehyde for the cross-linking of GNFs. Dextran aldehyde (DA) from dextran of molecular weight ranging from 35,000–45,000 could be dissolved in ethanol in presence of minimum quantity of aqueous borax. Nanofibres were cross-linked by dipping in this medium and cross-linked nanofibres maintained the fibrous morphology even after keeping in contact with water.

2. Materials and methods

2.1. Chemicals

Type A gelatin from porcine skin (300 bloom), dextran from leuconostoc mesenteroides with average molecular weight ranging from 35,000 to 45,000, sodium tetraborate decahydrate (borax), trinitro benzene sulphonic acid (TNBS), minimum essential medium, propidium iodide (PI), fluorescein diacetate (FDA), 3-(4,5-dimethylthiazol-2yl)-2,5,diphenyltetrazolium bromide (MTT), Hoechst 33258, glutaraldehyde and paraformaldehyde were purchased from Sigma Aldrich, Saint Louis, USA. Foetal bovine serum, trypsin, rhodamine-phalloidin etc., were obtained from Gibco (Invitrogen, India). Sodium metaperiodate, sodium chloride, disodium hydrogen phosphate, sodium dihydrogen phosphate, hydroxyl amine hydrochloride and acetic acid (AR grade) were procured from Merck (Mumbai, India) and used without further purification. Cellulose dialysis tubing with MWCO 3,500 from Spectrum Laboratories Inc.CA, USA was used. Double distilled water was employed in all the experiments and Milli Q water (Millipore) was used for cell culture.

2.2. Electrospinning

Solution for electrospinning was prepared by dissolving gelatin (26%, w/v) in water/acetic acid mixture (8:2, v/v) at 40 °C. Electrospinning was performed by ESPIN-NANO electrospinning machine fabricated by Physics Instruments Co. Ltd, Chennai, India. It composed of a high voltage power supply (up to 50 kV), a programmable syringe pump and a rotating drum target. Solution for electrospinning was taken in a 5 ml plastic syringe (Dispovan) capped with a needle of $0.60 \times 25\,mm^2$. The syringe was placed in the syringe pump aligned vertically to the drum collector. Electrospinning was performed with an applied voltage of 20 kV, tip to collector distance of 17 cm, and a flow rate of 0.3 ml/h at room temperature. Electrospun mats were collected over 0.5 mm thick aluminium foil wound over the rotating collector. These fibrous mats were kept for drying in a vacuum oven prior to cross-linking.

2.3. Preparation of cross-linking agent: periodate oxidation of dextran

DA was prepared by periodate oxidation of dextran with sodium meta periodate ($NaIO_4$) according to the procedure reported by Domb et al. (Sokolsky-Papkov, Domb, & Golenser, 2006). In brief, 2.66 g of $NaIO_4$ was dissolved in 10 ml of water containing 1 g of dextran and the reaction mixture was stirred under dark for 6 h at 25 °C. Resulting solution was dialyzed against double distilled water for 3 days by changing water until it was free from periodate. Samples were stored at −20 °C in deep freezer and thereafter dried by lyophilization. Molecular weight and polydispersity of dextran and DA were measured using Gel Permeation Chromatography (GPC). Samples at a concentration of 0.01 mg/ml were eluted with 0.02 M sodium nitrate solution through an ultrahydrogel column (Waters ultrahydrogel columns 2000/1000/500 in series) at a flow rate of 1 ml/min. Dextran standards of Mp 3,44,000, 21,100 and 9600 were used for relative calibration.

2.4. Aldehyde content and degree of oxidation in DA

Aldehyde content and degree of oxidation were estimated by chemical analysis. Estimation was based on the principle that the aldehyde group in DA would react with hydroxyl amine hydrochloride resulting in the release of hydrochloric acid (HCl). One mole of HCl would be released per mole of aldehyde group reacted. Amount of HCl released was estimated by titrating against standard sodium hydroxide (NaOH) solution. To estimate the aldehyde content in DA, 0.10 g of DA was dissolved in 25 ml of 0.25 N hydroxyl amine hydrochloride prepared in distilled water. Two drops of methyl orange indicator (0.05% solution) were added and allowed to stand for 2 h. The mixture was then titrated against 0.1 N NaOH taken in the burette. At the end point, the colour of the solution changed from red to yellow and the number of moles of NaOH reacted was calculated. This is equivalent to the number of moles of aldehyde groups present in the sample. From the number of moles of aldehyde groups obtained, percentage degree of oxidation was calculated. The estimation was performed in triplicate and the average values for degree of oxidation and aldehyde content are reported.

2.5. Cross-linking of electrospun GNFs

DA (0.05 g) was taken in 10 ml of pure ethanol and was stirred for 24 h. A minimum amount of borax solution (300 μl, 0.015 M) was added and again stirred for 3 h for complete dissolution. Even though the presence of borax will enhance the cross-linking efficiency (Balakrishnan et al., 2013), the amount was restricted to avoid precipitation in ethanol. Cross-linking was carried out by immersing 0.5 g of GNF mats in 10 ml of DA solution at 37 °C for 1, 3 and 5 days. These cross-linked mats are labelled as DA-GNF-1D,

DA-GNF-3D and DA-GNF-5D where 1D, 3D and 5D represent duration of cross-linking (1,3 and 5 days respectively).

2.6. Characterizations

Morphology and microstructure of the nanofibrous mats were examined using scanning electron microscope (SEM, FEI Quanta FEG200). Diameter distributions of the samples were determined using image analysis software (ImageJ) by randomly selecting 100 data points. Fourier transform infrared spectra (FTIR) were obtained (Spectrum 100, Perkin Elmer, USA) with universal attenuated total reflectance accessory (UATR) using standard ZnSe crystal at a light path angle of incidence of 45°. For each spectrum, 32 scans were accumulated at 4 cm^{-1} resolution in the scanning range of 4000–650 cm^{-1}. X-ray photoelectron spectroscopy (XPS) measurements were conducted (Ultra axis, Kratos analytical, Shimadzu, Japan) using monochromatic aluminium Kα X-rays. Thermogravimetric analysis (TGA) of as spun and cross-linked samples were carried out by TA instruments (Q 50 Thermogravimetric analyser, TA instruments, USA) using platinum pan in nitrogen atmosphere. Heating was performed from room temperature to 500 °C at a heating rate of 10 °C/min. Differential scanning calorimetry (DSC) was performed in TA instruments (Q 20 differential scanning calorimeter, TA instruments, USA) from –20 °C to 280 °C at a heating rate of 10 °C/min. Thermograms recorded were analyzed by TA Universal Analysis software. Mechanical properties of nanofibrous mats were determined by Universal Testing Machine (Instron 5050, Instron USA) with a load cell of 100 N capacity. Specimens were cut in the form of rectangular strips ($6 \times 0.4\ cm^2$) with an average thickness of 0.2 mm, and tested at a crosshead speed of 10 mm/min. Stress at break, Young's modulus as well as strain at break were measured based on stress-strain curve.

Swelling behaviour of as spun and cross-linked mats was examined in double distilled water at 37 °C. Weights of the dry and swollen mats were determined at different time intervals, up to 48 h. Swelling ratio was calculated using the following equation:

$$SR(\%) = ((W_s - W_d)/W_d) \times 100$$

(Where SR is the water absorption (%wt) of the fibrous mat, and W_d and W_s are the weights of the samples in the dry and swollen states, respectively).

TNBS assay was used to determine the degree of cross-linking of nanofibrous mats (Sheu, Huang, Yeh, & Ho, 2001). About 2 mg each of as spun and cross-linked mats were treated separately with 1 ml of 4% sodium bicarbonate solution and 1 ml of 0.5% freshly prepared TNBS solution. The mixture was heated at 40 °C for 2 h. To this mixture, 3 ml of 6 N HCl was added and temperature was raised to 60 °C. The solubilisation of cross-linked mats was achieved within 1 h. The resulting solution was diluted with 5.0 ml of deionized water, and the absorbance was measured spectrophotometrically (Agilent Technologies, Cary100 UV-Visible Spectrophotometer, USA) at 346 nm. The degree of cross-linking was calculated as follows.

$$\text{Degree of cross-linking} = 1 - \{(\text{absorbance}_{cl}/\text{mass}_{cl}) \times (\text{absorbance}_{ucl}/\text{mass}_{ucl})^{-1}\}$$

The subscripts cl and ucl stand for the cross-linked and uncross-linked GNFs.

In vitro degradation of as spun and cross-linked mats was evaluated in phosphate buffered saline (PBS). Previously weighed and dried samples were immersed in 5 ml of PBS at 37 °C for time periods ranging from one week to five weeks. Degradation behaviour of glutaraldehyde cross-linked gelatin nanofibres (GTA-GNF) was also studied for a comparison with DA cross-linked mats (Fig. S2, Supporting information). Experiment was performed in aseptic conditions. Different sets of samples were used for each week. After the predetermined time, medium was removed and weights of the samples were measured after lyophilization.

$$\text{Weight ratio} = \frac{W_2}{W_1}$$

W_1 and W_2 are the initial and final sample weights, respectively.

2.7. In vitro cytotoxicity

An *in vitro* cytotoxicity test by direct contact method was performed on DA cross-linked mat (DA-GNF-5D) with high density polyethylene (HDPE) and stabilized poly vinyl chloride (PVC) as negative and positive controls respectively. Mouse subcutaneous fibroblasts (L-929 cell line, American Type Cell Culture Collection) was maintained in Minimum Essential Medium (MEM) supplemented with 10% Foetal Bovine Serum, 100 IU/ml Penicillin and 100 μg/ml Streptomycin in a CO_2 incubator (Sanyo, Japan) set at 37 °C, 5% CO_2 and >90% relative humidity. The cells were detached using trypsin and seeded in a 24 well plate at a density of 1×10^5 cells per well. When the cells reached subconfluency, triplicate samples of DA-GNF-5D, negative control and positive control having approximate size of 10% of cell area were placed on the cells. The cells were incubated inside CO_2 incubator for 24–26 h. Cytotoxicity was assessed by observing the cells around test and control samples under phase contrast microscope (Motic AE31, Hong Kong).

2.8. Cell adhesion, viability and proliferation on DA-GNF-5D mats

Cells were allowed to adhere and grow on DA-GNF-5D mats for 48 h and then processed for analyzing cell adhesion and morphology. The mats were seeded with L-929 cells at a density of 5000 cells/cm^2 and cultured for 48 h. Cells cultured on cover glass were considered as control.

The viability of cells adhered on the scaffold was examined by incubating with FDA (10 μg/ml in serum free medium) for 5 min followed by treatment with PI (1 μg/ml) for 5 min. Stained samples were observed under fluorescence microscope (Leica DMIL, 6000B, Germany) using the filter cubes designated for FDA (I3) and PI (N 2.1).

The cell adhesion was analyzed by visualizing the morphology of adhered cells by staining the actin cytoskeleton structures. L-929 cells were seeded on DA-GNF-5D mats and control glass cover slips as described above and cultured for 48 h. Cells were fixed using 4% paraformaldehyde for 2 h and rinsed with PBS. The cell membrane was permeabilized by treating with 0.1% Triton-X 100 for 1 min and actin filaments were stained by incubating the samples with rhodamine-phalloidin (1:1000 dilutionin PBS) for 15 min. The cell nucleus was counter stained using Hoechst 33258 dye (0.1 μg/ml in PBS). The cell adhesion was observed under fluorescence microscope using Leica filter cubes specified for Hoechst (A) and rhodamine (N2.1).

Cell proliferation was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Samples (0.5 cm × 1 cm) were sterilized with 70% ethanol for 15 min followed by washing with 1×PBS for three times. Trypsinized mouse fibroblast cells (L929) were seeded on the samples (2000 cells/sample) and incubated for specific time period under standardized conditions (humidified incubator with 37 °C and 5% CO_2). After every 72 h, the cell culture medium was replenished with fresh one.

After 3rd and 5th day, the samples were washed with PBS solution and incubated with MTT solution (0.5 mg/ml) for 4 h at 37 °C. After 4 h the MTT solution was replaced with DMSO solution to dissolve the formazan crystal. Spectrophotometric analysis of the DMSO solution was done using ELISA reader (BioradiMark) at 595 nm.

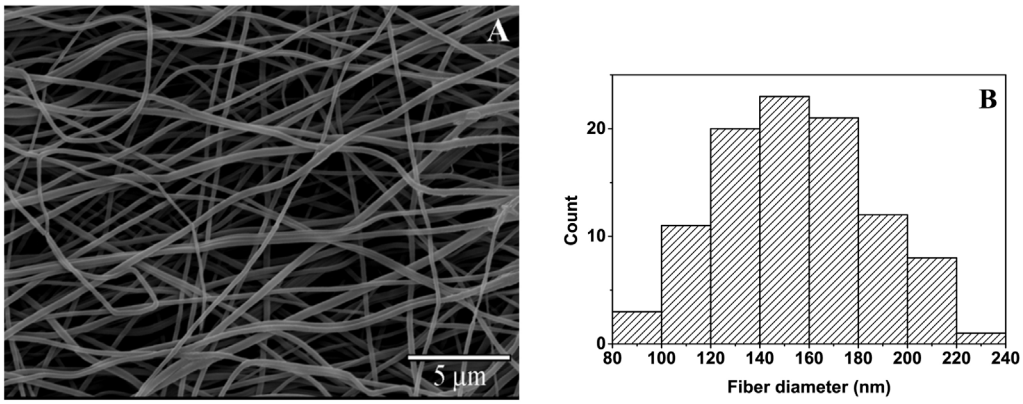

Fig. 1. (A) SEM micrograph of as spun fibrous mat using 26% (w/v) gelatin solution and (B) fibre diameter distribution of as spun nanofibres.

2.9. Statistical analysis

The results were expressed as mean ± standard deviation. Statistical analysis was performed by Student's *t*-test with $p < 0.05$ considered as being statistically significant.

3. Results and discussion

3.1. Fabrication of GNFs

Gelatin undergoes thermo reversible gelation in water at a temperature less than 35 °C which is the sol–gel transition temperature of gelatin. Hence, electrospinning of gelatin in water cannot be realized at room temperature. Recently researchers have started using solvent systems consisting of acetic acid and water (60% or 90%) for electrospinning of gelatin. However, it has been reported that high concentration of acetic acid in water (6:4 (v/v) acetic acid/water) can cause degradation of gelatin (Panzavolta et al., 2011). In the present work, electrospinning was attempted with minimum possible concentration of acetic acid and we could obtain smooth and fine nanofibres using 8:2 water/acetic acid mixture using 26% (w/v) gelatin solution (Fig. 1A). Presence of 20% acetic acid in the solvent system was essential to prevent gelation. Fig. 1B shows the fibre diameter distribution of the nanofibres obtained by measuring the diameters of randomly selected 100 data points.

3.2. Preparation of DA by periodate oxidation

DA was prepared by the controlled addition of sodium metaperiodate into dextran solution. The aldehyde content and degree of oxidation were measured by hydroxylamine hydrochloride reaction with DA and subsequent titration with NaOH. Table 1 shows the degree of oxidation and aldehyde content in DA. From the GPC analysis, the weight average molecular weights and polydispersities of dextran and DA were obtained (Table 1). It can be seen that there is not much variation in the weight average molecular weights (Mw's) of dextran and DA. Polydispersity of DA is higher (PDI = 3.5) than dextran (PDI = 1.86) as expected due to the excess amount of periodate used for the oxidation (Sokolsky-Papkov et al., 2006).

Table 1
Properties of dextran and DA.

Properties	Dextran	DA
Degree of oxidation (%)	0	87 ± 2%
Aldehyde content (mol/g)	0	$10.7 \pm 0.25 \times 10^{-3}$
Weight average molecular weight (M_w)	37,352	36,489
Polydispersity	1.86	3.5

3.3. Cross-linking and characterizations of GNFs

When in contact with water, GNFs lose the fibrous morphology completely, due to the high solubility of gelatin in water (Fig. S1B Supporting information). It has been found that electrospun fibres gradually form point bonds at the fibre junctions if placed in a high humidity (80–90%) for a certain period of time (Zhang et al., 2006). Researchers have adopted different cross-linking approaches to overcome these issues. In the present work, we employed a polysaccharide into which aldehyde functional groups were introduced by periodate oxidation as the cross-linking agent. Polysaccharide aldehydes are soluble only in aqueous solutions (either in PBS or in borax solution). Here, the prepared DA was dissolved in ethanol containing minimum quantity of aqueous borax solution. Addition of excess borax would cause it to be precipitated from ethanol. Thus, we ensured the dissolution of DA keeping the amount of water to be as minimum as possible. Nanofibrous mats were kept immersed in this cross-linking medium for 1, 3 and 5 days. The effect of cross-linking was examined by keeping the mats in aqueous medium for 1 h. It was found that the sample cross-linked for 1 day became transparent and sticky, while the samples cross-linked for 3 and 5 days remained intact. Fig. 2 shows the photograph of DA-GNF-5D and as spun mats immersed in water.

Morphological analysis of the fibres was carried out by SEM. The morphologies of cross-linked and swelled mats are shown in Fig. 3. The images reveal that the samples have undergone cross-linking and maintained the fibrous structure after cross-linking treatments. The DA-GNF-5D exhibited better morphology after

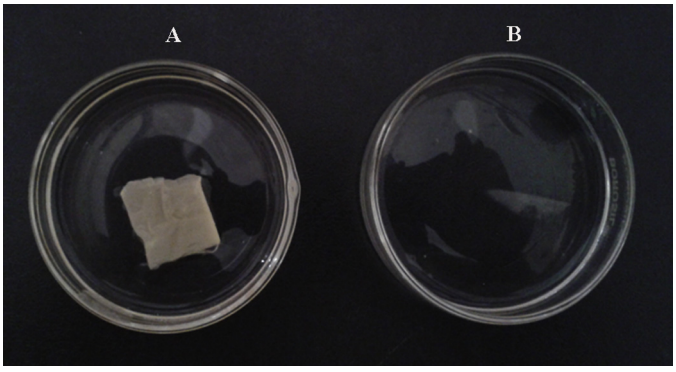

Fig. 2. (A) DA-GNF-5D (B) as spun GNF mats in water medium.

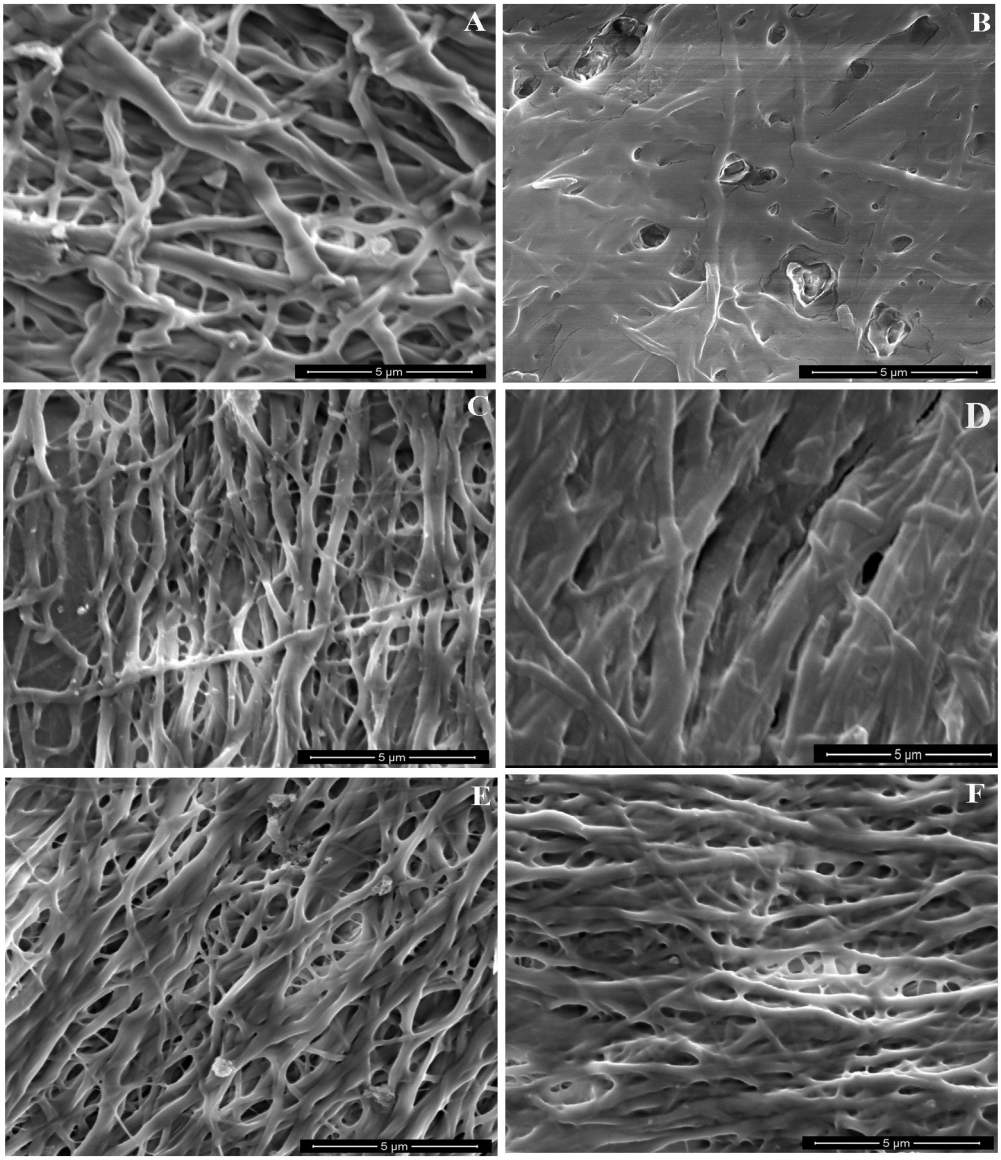

Fig. 3. SEM microstructures of (A) DA-GNF-1D, (B) DA-GNF-1D swelled in water, (C) DA-GNF-3D, (D) DA-GNF-3D swelled in water, (E) DA-GNF-5D and (F) DA-GNF-5D swelled in water for 24 h.

swelling in water (Fig. 3E) compared to the DA-GNF-1D and DA-GNF-3D (Fig. 3B and D). It can be seen that, swelling the mats in water causes fibres to fuse together in case of DA-GNF-1D and DA-GNF-3D mats whereas DA-GNF-5D mat shows discrete fibrous morphology. However, fibre diameter was found to increase as a result of cross-linking treatment and increased further after swelling in water (see Table 2). Diameter of the fibrous component is an important parameter, when considering the material as scaffold for tissue regeneration. The diameter of electrospun GNFs before cross-linking was 156 ± 30 nm. The diameters of electrospun DA-GNF-5D were 220 ± 65 nm and 286 ± 90 nm before and after swelling, respectively. Such small size fibres could physically mimic the structural dimension of the extracellular matrix of various native tissues and organs, which are deposited and proliferated on essentially fibrous structures ranging from nanometres to micrometres. Human cells can attach and organize well around fibres with diameters smaller than those of the cells. Hence, the fibrous scaffolds prepared from electrospinning can be considered as ideal candidates as matrices for tissue regeneration (Xu, Inai, Kotaki, & Ramakrishna, 2004; Zhang et al., 2006).The cross-linking of GNFs occurred by Schiff's base reaction of amino group of gelatin with aldehyde group of DA. The cross-linking reaction was found to progress slowly as evident from the required time of at least 5 days for maintaining the fibrous morphology in aqueous media. The cross-linking reaction between amino group of gelatin and aldehyde group of polysaccharide in presence of aqueous borax is reported to be very fast where both the reacting species were dissolved in aqueous medium (Balakrishnan & Jayakrishnan, 2005). However, in the present work, gelatin is in solid phase and the cross-linking agent, dextran aldehyde is in ethanolic medium resulting in the prolonged time required for cross-linking.

Table 2
Fibre diameters of GNF and DA-GNF mats before and after swelling in water. Mean ± standard deviations are reported.

Electrospun mats	Diameter (nm)	
	Before swelling in water	After swelling in water
As spun gelatin mats	156 ± 30	No fibrous structure
DA-GNF-1D	258 ± 90	389 ± 100
DA-GNF-3D	225 ± 70	359 ± 100
DA-GNF-5D	220 ± 65	286 ± 90

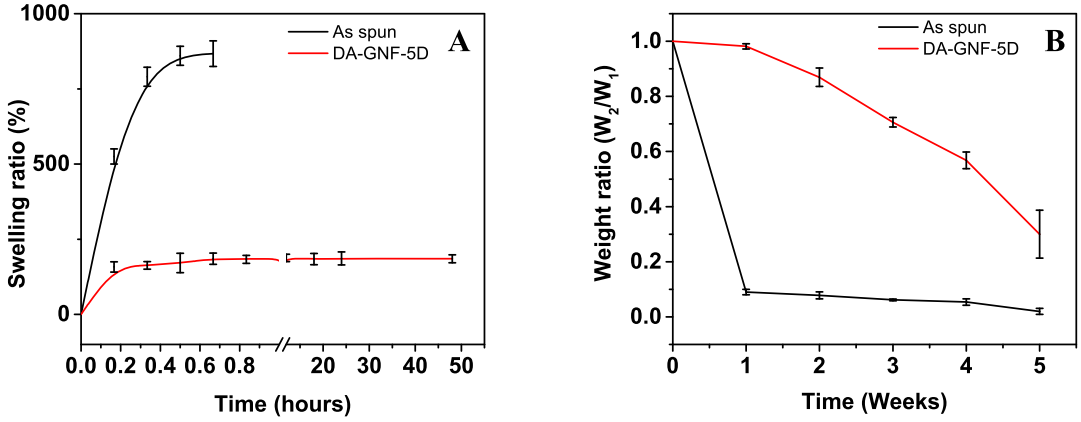

Fig. 4. (A) Swelling characteristic and (B) degradation behaviour of as spun and DA-GNF-5D mats.

Cross-linking of GNFs with genipin has been carried out by dipping the nanofibrous mat in ethanolic solution of genipin (Panzavolta et al., 2011). Even though, genipin is a small molecule which can penetrate into the fibrous mats easily, effective cross-linking was achieved after dipping the nanofibrous mats for 5–7 days. DA is a large molecule and cross-linking would have happened on the surface of the nanofibres. Here, effective cross-linking could be achieved by dipping the fibrous mats for 5 days in the cross-linking medium. Hence, 5 day cross-linked mats (DA-GNF-5D) were selected for further characterizations.

The extent of cross-linking was estimated by measuring the unreacted ε-amino groups in 5 day cross-linked and uncross-linked GNFs using TNBS assay. The degree of cross-linking was found to be $42 \pm 2\%$. Cross-linking of GNFs with glutaraldehyde and genipin have resulted in higher cross-linking degrees (~90%)(Panzavolta et al., 2011; Wu et al., 2011). The lower cross-linking degree obtained in the present work can be justified on the basis of the macromolecular nature of the cross-linking agent employed.

The swelling ability of a scaffold in water is an important aspect for its suitability for various tissue engineering applications. The swelling behaviour of 5 day cross-linked and uncross-linked electrospun mats were studied and the results indicated the effect of cross-linking on the swelling properties. These results demonstrated that swelling ratio of cross-linked gelatin is significantly lower than that of uncross-linked gelatin mat (Fig. 4A). Swelling of electrospun mats was reduced due to the cross-linking treatment. The higher swelling ratio of as spun gelatin is due to the large amount of water absorption of the highly porous structure of the fibrous mats. The experiment could not be performed for as spun mat beyond 40 min because of the dissolution. The cross-linked mat attained equilibrium swelling within 20 min.

The degradation behaviour of the nanofibres was evaluated by examining the weight loss of nanofibres with time in PBS at 37 °C. Fig. 4B shows the degradation behaviour of as spun and DA-GNF-5D mats. As spun mats got dissolved completely in the first week itself, the cross-linked mat was found to be stable for one week and thereafter showed a linear decrease in weight up to four weeks. The complete dissolution of the mat occurred at the end of five weeks demonstrating the degradability of the nanofibrous mats in the physiological pH. A comparison of degradation behaviour of DA-GNF-5D with GTA-GNF was also carried out. It was found that the latter exhibited a similar behaviour as that of DA-GNF-5D (Fig. S2, Supporting information). Gradual degradation of DA-GNF-5D mats with time would make them suitable matrices as tissue engineering scaffolds. The hydrolytic susceptibility of Schiff's base and the biodegradability of gelatin and DA cause the degradation and dissolution of cross-linked nanofibres. For individual nanofibres, the degradation profiles mainly determined by the polymer itself as hydrolysis of the polymer backbone is believed to be the usual mechanism. Gelatin contains random coil structure (amorphous) and some triple helical region (crystalline) also. In the absence of an enzyme, water penetrates the surface of nanofibre and preferentially attacks the amorphous regions, converting the long polymer chains into shorter and eventually water-soluble species. Since the crystalline regions are still intact, the nanofibre does not fall apart. As hydrolysis continues, the nanofibre eventually starts to disintegrate and disappear (Liu, Thomopoulos, & Xia, 2012).

The effect of the DA cross-linking on the chemical composition on the surface of gelatin mat was determined by XPS measurements. Table 3 gives the atomic composition of gelatin and DA-GNF-5D surfaces obtained from high resolution XPS spectra. The results show that DA-GNF-5D exhibits higher values compared to GNF for C/N ratio (7.3 versus 3.59) and O/N ratio (2.7 versus 1.29). This is due to the fact that during cross-linking, polysaccharide moieties are introduced on gelatin via imine bond formation by the reaction between aldehyde groups of DA and primary amino groups of gelatin. As a result, percentage atomic composition of carbon and oxygen increases, enhancing the C/N and O/N ratio. This indicates the incorporation of DA into the gelatin network and shows the presence of DA moieties on the surface of the mats. Survey scan spectrum of GNF, DA and DA-GNF-5D (Fig. 5) also shows the incorporation of DA into GNF after cross-linking reaction. The decrease in intensity of N1s peak showed the relative increase in the C and O atoms in the DA-GNF-5D mat.

FTIR spectra of dextran, DA, as spun and cross-linked GNFs are given in Fig. 6A. The spectrum of DA showed peaks at 1739 cm^{-1} and 2852 cm^{-1} corresponding to carbonyl —C=O and aldehydic C—H stretching respectively. DA-GNF-5D showed the characteristic peaks of gelatin as well as dextran. The presence of dextran moieties in the cross-linked GNFs can be inferred from the characteristic C—O—C stretching band of dextran observed at 1012 cm^{-1} in the spectrum of DA-GNF-5D.

TGA and DSC analysis were performed on as spun and DA-GNF-5D in order to investigate the effect of cross-linking on thermal stability of the nanofibres. TGA thermograms and its first order derivative curves (DTG) of as spun and DA-GNF-5D mats from 25 °C to 500 °C are presented in Fig. 6B. The TGA plot of pure gelatin in

Table 3
Elemental composition of GNF, DA and DA-GNF-5D from high resolution XPS spectra.

Element	GNF (%)	DA-GNF-5D (%)	DA (%)
C	61	66	57
N	17	9	0
O	22	25	43

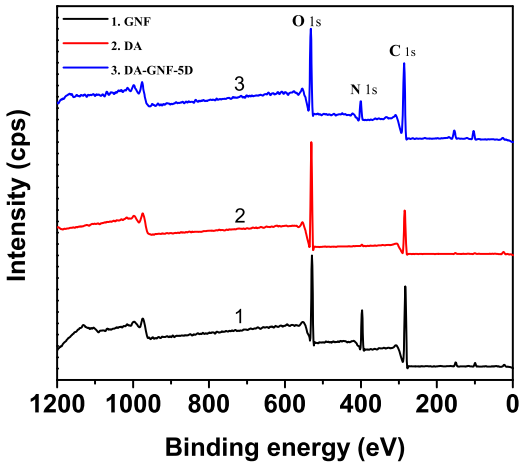

Fig. 5. XPS survey scans spectra of GNF, DA and DA-GNF-5D mats.

this range normally displays two thermal stages, i.e. loss of water, between 25 and 100 °C and gelatin decomposition, between 250 and 450 °C. DTG thermograms show that as spun mat exhibits maximum decomposition at 291 °C while it has been shifted to 304 °C in the case of cross-linked mat demonstrating an improved thermal stability upon cross-linking. DSC thermograms of as spun and DA-GNF-5D mats in the range of −20 to 250 °C were carried out in order to identify the effect of cross-linking on the thermal transitions of GNFs. The DSC thermogram of gelatin exhibited two endothermic peaks corresponding to helix to coil transition in the range of 90–110 °C and decomposition of gelatin in the range of 200–230 °C. On comparing the DSC curves of as spun and DA-GNF-5D mats, it is found that the denaturation temperature of as spun mat is 88 °C which is shifted to 101 °C after cross-linking (Fig. 6C). This observation confirms that the cross-linked mats have higher thermal stability than that of as spun fibres. Zhang et al. (2006) have reported similar thermal behaviour for glutaraldehyde cross-linked GNFs.

Table 4
Mechanical behaviour of as spun and DA-GNF-5D mats.

Mechanical properties	As spun	DA-GNF-5D
Stress at break (MPa)	8.29 ± 0.53	30 ± 3.47
Strain at break (%)	8 ± 3	22 ± 16
Young's modulus (MPa)	394 ± 96	904 ± 68

The mechanical properties of GNFs cross-linked with DA were examined. Based on the stress-strain measurements of these membranes, tensile strength, Young's modulus and strain at break are summarized in Table 4. The stress-strain behaviour of as spun and DA-GNF-5D mats is shown in Fig. 6D. The results indicate that the crosslinking treatment significantly improved the mechanical performance of GNFs. After crosslinking, both the tensile strength and modulus were enhanced about three times than those of the un-cross-linked GNFs. The results suggest that remarkable improvement in the mechanical behaviour is achieved by treatment with DA. Covalent bonds formed along the GNFs by the Schiff's base reaction with DA are responsible for this tremendous improvement.

Nanofibrous gelatin mats of tensile moduli 20–100 MPa have been investigated for hard and soft tissue engineering applications (Panzavolta et al., 2011; Zhang et al., 2006). Mechanical properties as well as degradation behaviour of the nanofibrous scaffold can be tuned by the type of the cross-linking agent and cross-linking time. The properties can also be tailored by incorporating various synthetic polymers such as poly (L-Lactide) (An, Liu, Guo, Kumar, & Wang, 2010)poly (caprolactum) (Zhang, Huang, Xu, Lim, & Ramakrishna, 2004), poly aniline (Li et al., 2006) etc. Controlling

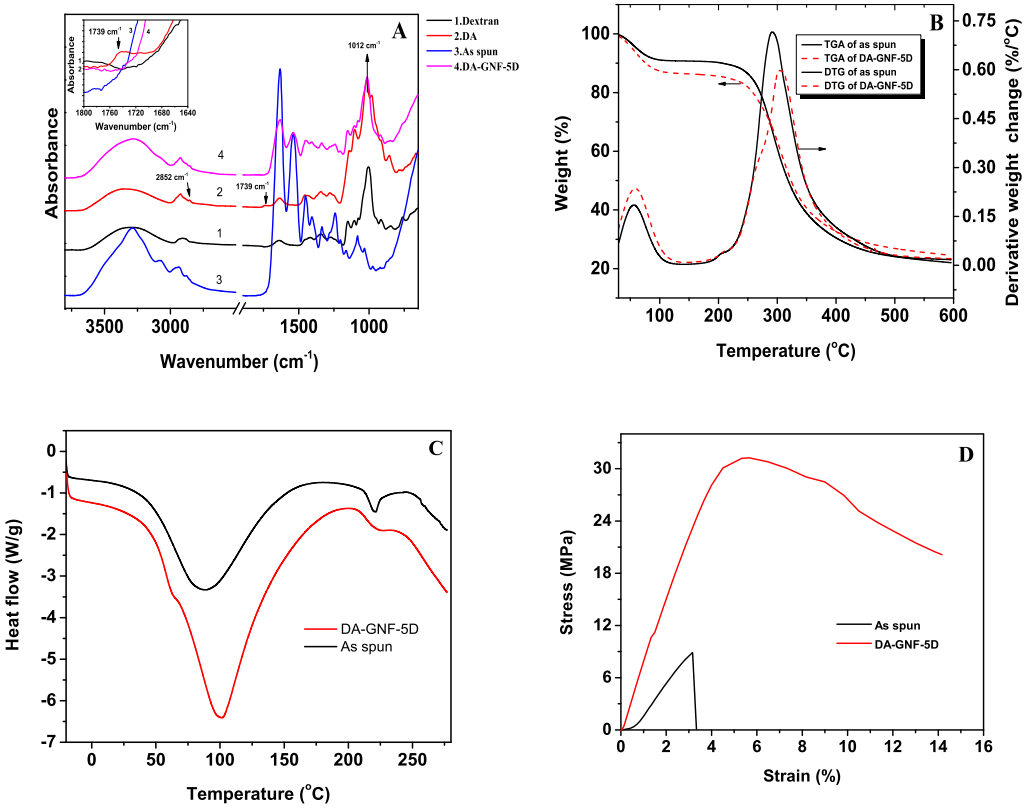

Fig. 6. (A) FTIR spectra, (B) TGA thermogram, (C) DSC thermogram and (D) mechanical behaviour of as spun mat and DA-GNF-5D mats.

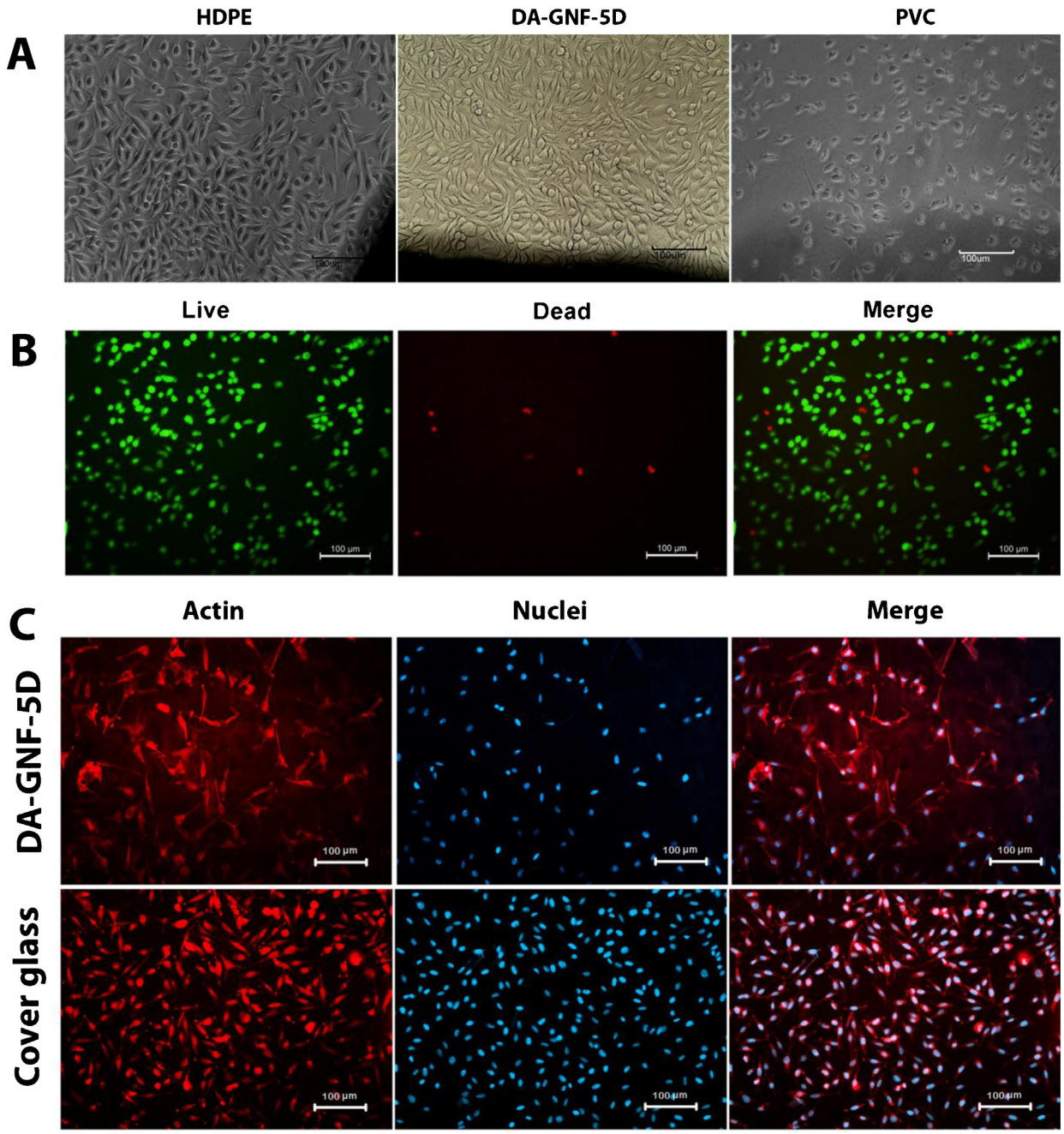

Fig. 7. (A) Light microscopic images of L929 cells on HDPE control, DA-GNF-5D mats and PVC disc after 24 h contact. (B) Fluorescence microscopic images of live and dead cells on DA-GNF-5D mats obtained by FDA-PI staining. (C) Actin cytoskeleton and nucleus staining of L-929 cells using rhodamine-phalloidin (red) and Hoechst 33258 (blue) on DA-GNF-5D mat and cover glass. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.) .

the mechanical properties and degradation behaviour of the nanofibres will help to optimize the scaffolds for regeneration of a specific tissue type.

3.4. *In vitro* cytotoxicity, cell viability and proliferation

Cytotoxicity test methods help in screening materials that are intended to be used in medical devices. *In vitro* cytotoxicity tests with mammalian cells serve as the first step in evaluating biocompatibility of a biomaterial. *In vitro* direct contact cytotoxicity test using L-929 fibroblast cells with DA-GNF-5D showed non cytotoxic reactivity to fibroblast cells after 24 h contact. The cells around DA-GNF-5D mats maintained the characteristic spindle morphology without causing any toxic responses like cell detachment, lysis etc. (Fig. 7A). The negative control (HDPE) showed non-cytotoxicity and positive control (PVC) showed severe cytotoxicity to L-929 cells. Comparison with negative and positive controls confirmed the non-cytotoxicity of DA-GNF-5D samples.

Most of the tissue derived cells are anchorage dependent that require a surface to adhere and grow. Hence cell adhesion on biomaterial is of much interest as it is the initial event that is followed by many critical processes such as attaining morphology, spreading, migration and cell function. Cells were allowed to adhere and grow on DA-GNF-5D mats for 48 h and viability of the cells on the fibrous mat was ascertained by live-dead staining. Viable cells are observed as green and nucleus of dead cells stained red. The fluorescence microscopic image (Fig. 7B) showed that, cells are viable on DA-GNF-5D mats.

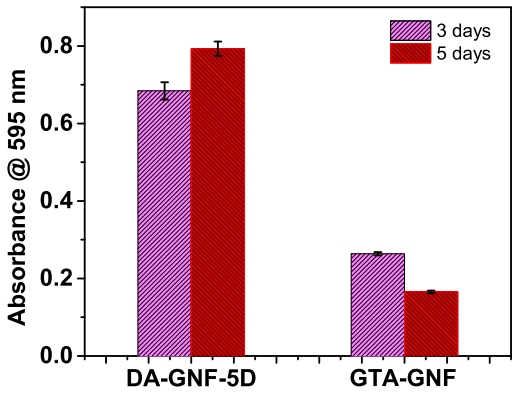

Fig. 8. Cell proliferation on DA-GNF-5D and glutaraldehyde cross-linked (GTA-GNF) mats.

One of the key requirements in tissue engineering is ensuring robust adhesion between cell and biomaterial (Metcalfe & Ferguson, 2007). Spreading of cells on nanofibrous mats were analyzed by observing the attached cell morphology by fluorescence microscopy. Cells cultured on nanofibrous mats showed spread fibroblast morphology indicating good cell adhesion similar to cells on control cover glass (Fig. 7C)

Cellular activity and proliferation on DA-GNF-5D mat was studied *in vitro* by MTT assay. For a comparison, cellular activity and proliferation assay on GTA-GNF also performed. Fig. 8 indicates the activity of L-929 fibroblast cells on DA-GNF-5D mat and GTA-GNF for 3 and 5 days. The absorbance value increased during 3 to 5 days confirming better cellular viability and proliferation on DA-GNF-5D mat compared to GTA-GNF. In case of GTA-GNF mat, the absorbance value has come down on the 5th day indicating some toxic response of glutaraldehyde on cells. Statistical analysis of absorbance values obtained for 3 and 5 day for DA-GNF-5D mats showed that there was statistically significant difference between the two time points ($p < 0.05$).

4. Conclusions

GNFs were fabricated by electrospinning process using a solvent mixture of water and acetic acid keeping the acetic acid concentration as minimum as possible. The electrospun nanofibres could be effectively cross-linked with DA. The cross-linked nanofibres maintained the fibrous morphology after keeping in contact with water and exhibited a significant improvement in the mechanical behaviour. Preliminary *in vitro* cytotoxicity study of the nanofibres revealed that DA cross-linked gelatin mat is non-cytotoxic towards L-929 cells with good cell adhesion, viability and proliferation. These cross-linked nanofibres with gradual degradation behaviour under physiological conditions have a great potential as scaffolds for tissue engineering.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2014.08.023.

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