



Electrospinning of carboxymethyl chitin/poly(vinyl alcohol) nanofibrous scaffolds for tissue engineering applications

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ABSTRACT

A novel fibrous membrane of carboxymethyl chitin (CMC)/poly(vinyl alcohol) (PVA) blend was successfully prepared by electrospinning technique. The concentration of CMC (7%) with PVA (8%) was optimized, blended in different ratios (0–100%) and electrospun to get nanofibers. Fibers were made water insoluble by chemical followed by thermal cross-linking. *In vitro* mineralization studies identified the ability of formation of hydroxyapatite deposits on the nanofibrous surfaces. Cytotoxicity of the nanofibrous scaffold was evaluated using human mesenchymal stem cells (hMSCs) by the MTT assays. The cell viability was not altered when these nanofibrous scaffolds were pre-washed with phosphate buffer containing saline (PBS) before seeding the cells. The SEM images also revealed that cells were able to attach and spread in the nanofibrous scaffolds. Thus our results indicate that the nanofibrous CMC/PVA scaffold supports cell adhesion/attachment and proliferation and hence this scaffold will be a promising candidate for tissue engineering applications.

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1. Introduction

Polymeric fibers having diameters ranging from a few nanometers up to some microns can be successfully used for a wide variety of applications like reinforcements in nanocomposites (Huang, Zhang, Kotaki, & Ramakrishna, 2003), as nanowires, nanotubes (Hu, Odom, & Lieber, 1999), and in tissue engineering (Deitzel et al., 2002; Khil, Kim, Kim, Park, & Lee, 2004; Xu, Inai, Kotaki, & Ramakrishna, 2004). A number of processing methods, such as drawing (Jayaraman, Kotaki, Zhang, Mo, & Ramakrishna, 2004), self assembly (Hartgerink, Beniash, & Stupp, 2002), template directed synthesis (Hulteen, Chen, Chambliss, & Martin, 1997), phase separation (Ma & Zhang, 1999), and electrospinning are already developed to fabricate micro or nanoscale fibrous scaffolds (Lu et al., 2008). Electrospinning, a suitable technique for the production of small-diameter fibers, has been developed in the first half of the 20th century (Formhals, 1934), but only recently Reneker and coworkers have investigated the process in more detail demonstrating its versatility (Reneker & Chun, 1996; Reneker, Yarin, Fong, & Koombhongse, 2000). Briefly, in electrospinning fibers are deposited on the properly grounded target as a non-woven highly porous mesh (Mathews, Wnek, Simpson, & Bowlin, 2002). Placing a rotating collector between the needle and the grounded plate allows

deposition of the fibers on the collector with a certain degree of alignment (Deitzel, Kleinmeyer, Harris, & Beck Tan, 2001). The possibility to produce meshes with a high surface area makes electrospinning an ideal solution for applications which require seeding of cells, like vascular tissue engineering (Li, Laurencin, Catterson, Tuan, & Ko, 2002). Moreover, the architecture of the produced non-woven meshes is similar to that found in most natural extra-cellular matrices, thus enhancing the applicability of these scaffolds (Khil et al., 2004).

The science and technology of nanomaterials have attracted wide attention because of their superior functionalities due to their extremely large and active surface areas. Most of the biopolymers physically supporting living bodies, such as cellulose, chitin, and silk, form hierarchal structures in plants and animals that increase in size from the simple molecules and highly crystalline fibrils at the nanometer level to composites at the micron level upward. Thus, these biopolymers intrinsically have the potential to be converted to crystalline nanofibers by the so-called downsize processing, although generally, in biological bodies, these nanofibers are tightly bound to each other through an endless number of hydrogen bonds. Compared to nanofibers prepared from synthetic polymer solutions by bottom-up processing (e.g. electrospinning), these bionanofibers have remarkable advantages in terms of high crystallinity, reproducibility, biodegradability, and biocompatibility. Fabrication of chitosan nanofibers through electrospinning (Greiner & Wendorff, 2007; Li & Xia, 2004; Tekmen, Suslu, & Cöcen, 2008), has recently been extensively investigated due to potential

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biomedical applications such as engineering tissues and delivery of pharmaceutical agents (Duan et al., 2006; Hohman, Brenner, & Rutledge, 2001; Ignatova, Starbova, Markova, Manolova, & Rashkov, 2006; Pham, Sharma, & Mikos, 2006; Prabakaran & Jayakumar, 2009; Prabakaran, Rodriguez-Perez, de Saja, & Mano, 2007; Reneker et al., 2000; Shin, Zeng et al., 2003; Ziabicki, 1976; Hohman, Brenner, & Rutledge, 2001). Recently, chitosan-based nanofibers have been successfully electrospun from chitosan solutions blended with poly(ethylene oxide) (Bhattarai, Edmondson, Veisoh, Matsen, & Zhang, 2005), PVA (Jia, Gong, Gu, Kim, & Dong, 2007; Zhou, Yang, & Nie, 2006), or silk fibroin (Park, Jeong, Yoo, & Hudson, 2004). Also, electrospinning of homogeneous chitosan (Geng, Kwon, & Jang, 2005; Ohkawa, Cha, Kim, Nishida, & Yamamoto, 2004), or chitosan derivatives (Neamark, Rujiravanit, & Supaphol, 2006), has been also reported. However, some organic solvents or organic acids solvents such as 1, 1, 1, 3, 3, 3-hexafluoro-2-propanol (HFIP), chloroform, trifluoroacetic acid (TFA), acrylic acid, and acetic acid must be employed in the fabrication of these chitosan nanofibers. The trace toxic organic solvent or acid in electrospun products is harmful when it is applied to wounded human skin or tissue. To overcome these problems, in this research, the water-soluble carboxymethyl chitin (CMC) was electrospun from aqueous solution using PVA as the binder. Here, water is an ideal solvent for both fabrication processes and biomedical applications of CMC and PVA. Electrospinning carried out in neutral pH and the fibers thus obtained were cross linked with glutaraldehyde. The cytocompatibility of the scaffold for cell attachment and proliferation was performed using human mesenchymal stem cells (hMSCs).

2. Materials and methods

2.1. Materials

Carboxymethyl chitin (CMC) with viscosity 15 mPa s for 1% solution at 25 °C was purchased from Koyo chemicals Co. Ltd., Japan. Polyvinyl alcohol with degree of hydrolysis 89% (Viscosity 35–50 CPs for 4% solution at 20 °C) was purchased from Qualigens Fine Chemicals Pvt. Ltd, India. Calcium chloride and di-sodium hydrogen phosphate was purchased from sd-fine, India. Glutaraldehyde (Biochemica grade) was purchased from Fluka. Syringes and needles were purchased from BD Sciences Ltd Spain. All chemicals were used as received.

2.2. Preparation of electrospinning solutions

The CMC solutions with concentrations ranging from 3 to 9 wt% were prepared by dissolving 0.3, 0.5, 0.7 and 0.9 g of CMC in 10 ml distilled water for 1 h. PVA solution of 8 wt% was prepared by dissolving 0.8 g of PVA in 10 ml distilled water with moderate stirring at 60 °C for 2 h. To determine the most favorable spinnable concentration of CMC with PVA, each CMC solution was mixed with 8 wt% of PVA solution (in 1:3 ratio) with the help of a magnetic stirrer. The selected concentration of CMC, 7 wt% CMC solution, was mixed with 8 wt% PVA solution in seven different weight compositions such as 0/100, 20/80, 40/60, 50/50, 60/40, 80/20, and 100/0. All the solutions were mixed well in a beaker for 2 h to get a homogeneous mixture.

2.3. Electrospinning

Electrospinning was carried out at room temperature for all the concentrations of CMC/PVA. A custom-designed electrospinning setup was fabricated using a high voltage DC power supply (Model RR30P, 0–30 kV, Gamma High Voltage Inc., USA). The polymer solutions were delivered from a syringe pump KDS 220, (KD Scien-

tific Inc., USA) fitted with 10 ml syringes having blunt ended metal needles of 21 gauges. All the sample solutions were spun to a grounded aluminum target fixed on a wooden stand from the tip of the needle by applying a voltage of 10 kV. A distance of 8 cm was maintained between the aluminum target and spinneret.

2.4. Cross-linking studies

The CMC/PVA fibrous scaffold in 20:80 ratios was selected for cross-linking studies. In order to get the cross-linked nanofibers, all the fibrous mats were cut into 6 cm² pieces and allowed to keep under glutaraldehyde vapors for 12 h in a desiccator, followed by heating at 112 °C for 12 h. Water resistance of the scaffold was evaluated by immersing it in distilled water for different time intervals up to 48 h.

2.5. In vitro biomineralization and Energy Dispersive Spectrum (EDS) evaluation on CMC/PVA fibrous membranes

CMC/PVA-hydroxyapatite composite fibrous membranes were prepared by alternate soaking method (Madhumathi et al., 2009) 200 mM of CaCl₂ solution was prepared and its pH was adjusted to 7.4 by adding Tris-HCL and also 120 mM of Na₂HPO₄ aqueous solution was prepared. Scaffolds in the sizes of 1 cm² were soaked in 10 ml of CaCl₂ solution for 2 h at 37 °C. Once it was removed from the solution, the excess moisture on it was blotted using filter paper, followed by soaking it in 10 ml of Na₂HPO₄ for 2 h at 37 °C. This constituted one cycle. Similarly this experiment was continued with two more CMC/PVA fibrous membranes for 3 and 5 cycles. After washing, the samples were then dried at 37 °C. The mineralized fibrous scaffolds were characterized by Scanning Electron Microscopy (SEM). Elemental composition of thus formed calcium phosphate was evaluated by the Energy Dispersive Spectrum (EDS) attachment of JEOL analytical SEM.

2.6. MTT assay

The cytotoxicity of the electrospun fibers was evaluated based on a procedure adapted from the ISO10993-5 standard test method (indirect contact) (Yingshan et al., 2008). The viability of cells attached on the electrospun scaffold was quantitatively determined using the colorimetric MTT (Sigma) assay. This assay measures the reduction of the tetrazolium component of MTT by viable cells. Therefore, the level of the reduction of MTT into formazan can reflect the level of cell metabolism. For the MTT assay the electrospun samples cross linked with glutaraldehyde were autoclaved and were incubated with complete medium at 37 °C for 24 h with an extraction ratio of 6 cm²/mL. After the incubation period, the supernatant solution was removed and diluted with fresh complete media to get the extraction media samples in the concentrations of 10%, 25%, 50%, and 100% (relative to supernatant). hMSCs were seeded in 96-well plate at a density of 5×10^3 cells per well. After 24 h of incubation, the culture medium was replaced with the prepared extraction medium as mentioned above and was incubated for another 24 h. Then the MTT reagent was added to the wells and was incubated for another 4 h. Thereafter the formazan crystals formed were solubilized using the solubilizing buffer (Triton X-100, 0.1 N HCL and isopropanol). The absorbance of the samples was read at 570 nm in a spectrophotometer (Biotek).

2.7. Cell proliferation and spreading

CMC/PVA (20:80) electrospun samples were ethanol sterilized, washed in sterile PBS, dried and then transferred to 24 well plates under aseptic conditions. Samples were seeded with 5×10^4 cells/well on complete culture media (α MEM containing 20% serum

from selected lots and 50 U/ml antibiotics) and were incubated for 12, 24 and 48 h, in the CO₂ incubator under standard culturing conditions. Cell seeded constructs were taken out after desired period of incubation, rinsed twice with PBS to remove the non adherent cells, and subsequently fixed with 2.5% glutaraldehyde (Sigma) for 1 h. After that, the samples were rinsed in PBS, dehydrated through alcohol gradient and air dried overnight. Dry samples were sputter coated with platinum for the scanning electron microscopic evaluation of the cell attachment and spreading over time on the scaffolds.

2.8. Characterization techniques

The viscosity of the electrospinning solutions was measured using Brookfield viscometer (Model DV-|| + Pro). 10 ml of each well-mixed solutions was taken in the built in stainless steel container of the viscometer and measurements were done at 22 °C. In conductivity measurements, 5 ml of each solution was taken in a plastic container and measured for its conductivity using EUTECH COND610 conductivity meter. The microscopic structure of the electrospun fibers before and after immersion in water was examined using a JEOL analytical SEM. All the samples were sputter coated with platinum prior to SEM imaging using a JEOL auto fine coater (model JFC-1600) at 10 mA for 120 s. Spectroscopic evaluation of CMC, PVA and CMC/PVA fibrous samples was carried out using a FTIR (Perkin Elmer Spectrum RX1) in the range between 2000 and 500 cm⁻¹, with a resolution of 2 cm⁻¹.

3. Results and discussion

3.1. Electrospinning

Our initial studies for determining the optimum CMC concentration required for electrospinning was determined at four different concentrations of CMC (3%, 5%, 7% and 9%) with PVA. Electrospinning generally produces non-woven matrices with randomly arranged, ultrafine fibers with nanometer diameters. Apart from other concentrations, 7% CMC showed comparatively more uniform morphology and was found to be the distinctiveness of the chitin based polymer systems as reported earlier (Shin, Hohman, Brenner, & Rutledge, 2001). A massive difference in the fiber morphology and viscosity was found when the optimized CMC

concentration (7%) was mixed with PVA in different weight compositions. When electrospun, it was hard to get fibers for 100% of CMC, instead of fibrous structure, pure CMC gave globular drop-like deposition in solution phase on the collecting target (Fig. 1). Fiber formation efficiency was found to be increasing when PVA was blended with CMC. For CMC content of 20%, spinning dots rarely interconnected with fibrous structure were observed instead of fibers (Fig. 1a). A similar report was previously reported (Yingshan et al., 2008). The spinning dot morphology was changed to beaded morphology, when the concentration of PVA increased to 40% (Fig. 1b). This bead on string morphology indicates the initiation of fiber forming tendency of CMC with the increase of PVA concentration. Fiber with slightly different morphology from bead on string was observed when the concentration of PVA increased to 50% in the CMC/PVA mixture (Fig. 1c). A transition of bead on string morphology to spindle like morphology was observed for the 60% PVA concentration (Fig. 1d). Remarkable differences in the morphology of fibers were observed when the CMC concentration decreased to 20% (Fig. 1e). In this structure, all the fibers showed exactly the same structural similarity of fine nanofibers of 100% PVA as shown in (Fig. 1f).

3.2. Conductivity and viscosity measurements

The relation between viscosity and conductivity of all the seven compositions of CMC/PVA mixtures is shown in Fig. 2. The decrease of viscosity values from 1179 to 533 cP for 100–0% CMC clearly denotes the fiber formation ability of the solutions (Fig. 2a). The morphology of fibers and their diameters were strongly influenced by the composition of the solutions. Even the viscosity value of CMC solution was optimum to form nanofibers, conductivity value was unacceptably higher. That is, conductivity of pure CMC solution is very much higher than that of PVA. So, when the blend composition increased from 0% to 100% CMC, conductivity values of the corresponding solutions were also increased. As PVA concentrations increased, there were decreased surface tension and optimum conductivity values, which could impart the solution, a better environment for producing nanofibers. From literatures, it is also evident that the surface tension value of pure CMC is very high, which will prevent the fiber formation. All these factors as mentioned above govern the fiber formation ability of CMC/PVA solution at different compositions.

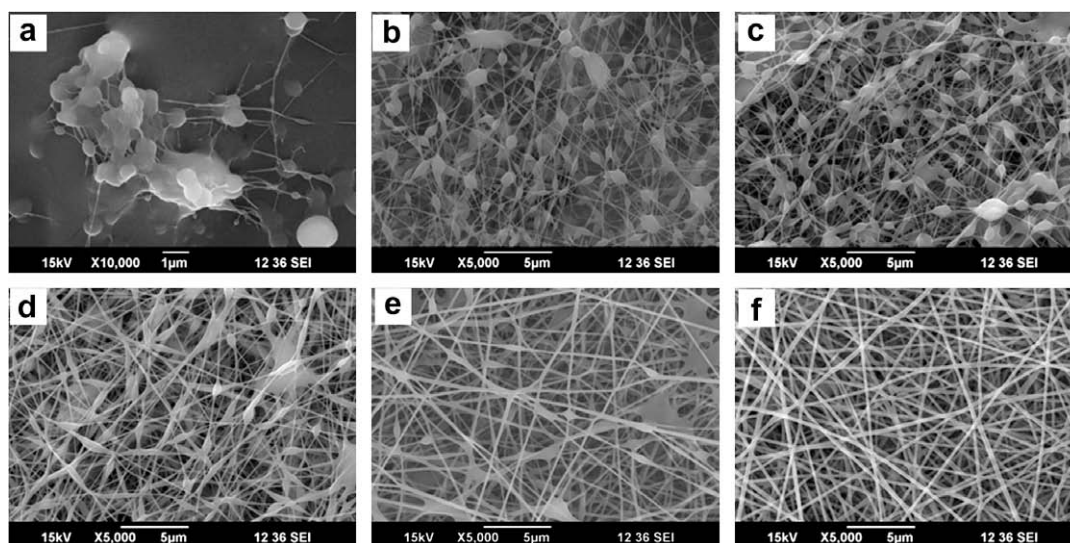


Fig. 1. Scanning electron micrographs of electrospun fibers of 7% CMC with 8% PVA in different combinations: (a) CMC/PVA = 80/20 (b) CMC/PVA = 60/40 (c) CMC/PVA = 50/50 (d) CMC/PVA = 40/60 (e) CMC/PVA = 20/80 and (f) CMC/PVA = 0/100.

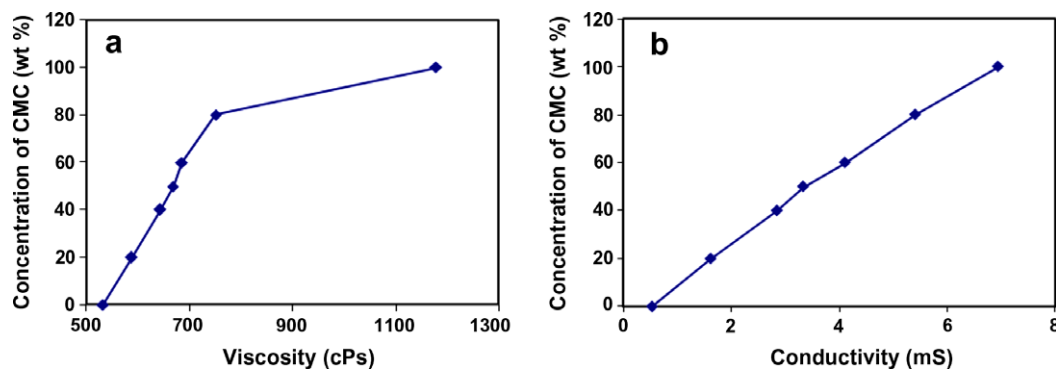


Fig. 2. Variation in (a) viscosity and (b) conductivity with respect to combination of CMC and PVA.

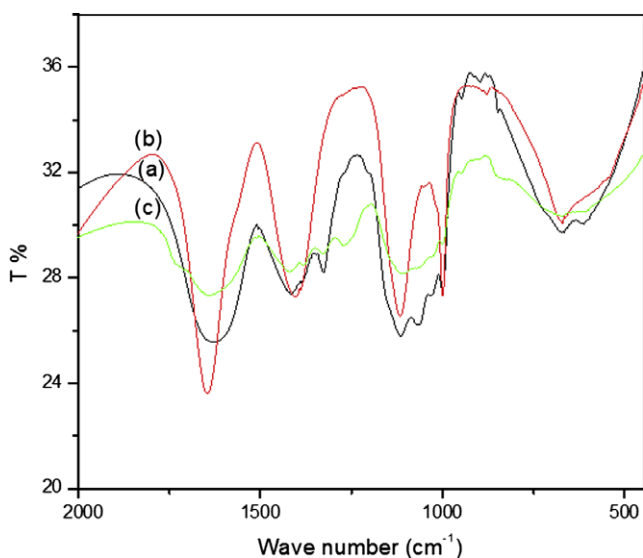


Fig. 3. FTIR spectra of CMC, PVA and CMC/PVA scaffolds: (a) PVA (b) CMC and (c) CMC/PVA.

3.3. FTIR studies

Fig. 3 shows the FTIR spectra of CMC, PVA and CMC/PVA (50/50) scaffolds. Two sharp peaks were obtained at 1653 and 1118 cm^{-1} and they were due to N–H bending and C–O stretching of CMC. Similarly, the peaks at 1613 and 1124 cm^{-1} represented the absorption values of PVA for N–H and C–O. But for CMC/PVA, as they mixed together, all the characteristic peaks became broader and showed the absorption values at 1608 and 1104 cm^{-1} . This shift in absorption could be due to the decreased electron density by van der Waals force of attraction or hydrogen bonding.

3.4. Cross-linking studies

Cross-linking studies were carried for the material to become insoluble in water. The SEM images were taken to analyze any morphological changes in the fibrous structure (Fig. 4). No remarkable morphological changes in fibrous structure were observed after exposure to glutaraldehyde vapors (Fig. 4b), followed by heating (Fig. 4c). The cross linking occurred in the fibrous scaffold by glutaraldehyde vapors and heating could be due to two different mechanisms. In first case, the O atom of each aldehyde group condenses with acetyl groups (NHCOCH_3) of two CMC chains to form C=N bonds giving acetic acid as byproduct. In the second case, instead of acetyl group, two hydrogen atoms from two OH groups of PVA condense with the O atom of each CHO group in glutaraldehyde to form two C–O linkages leaving water as the byproduct.

The solubility of cross-linked fibers in water was next determined by the SEM images. The fibers without cross-linking were first immersed in water. During 1 h of immersion, all the fibers were found dissolved due to the faster dissolution of water-soluble CMC and PVA nanofibers (Fig. 5a). But the cross-linked fibers showed no changes in fiber morphology even they immersed in water up to 48 h (Fig. 5b and c). This could be due to the increased resistivity of the fibers against water. The enhanced stiffness and rigidity developed by cross linking may be responsible for bringing such kind of increased resistivity.

3.5. *In vitro* mineralization and EDS analysis

The applicability of the fibers as bio-composite material was examined by *in vitro* mineralization studies. Alternate soaking method for three time intervals was used to deposit calcium phosphate on the surface of the fibrous scaffold. When the scaffold was immersed for 1 cycle, unlike the pure fibrous structure, surface of the fibers became partially coated with hydroxyapatite deposition (HAp) as shown by the SEM images. Fig. 6 shows the lower and higher magnification SEM images of the scaffolds immersed for 1

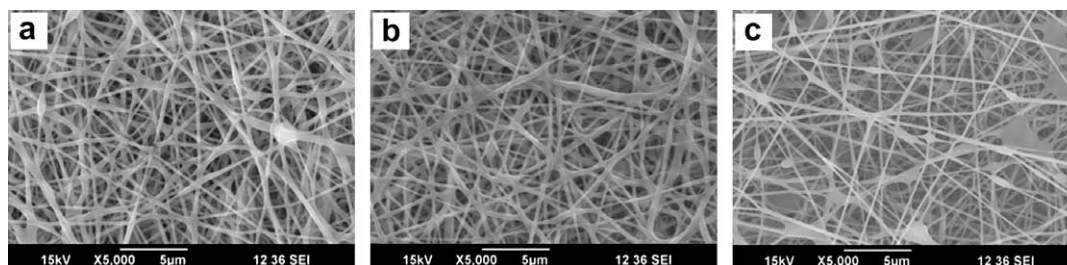


Fig. 4. SEM images of the electrospun fibers before and after cross linking: (a) As spun fiber (b) After exposure with glutaraldehyde and (c) Exposure with glutaraldehyde followed by heating.

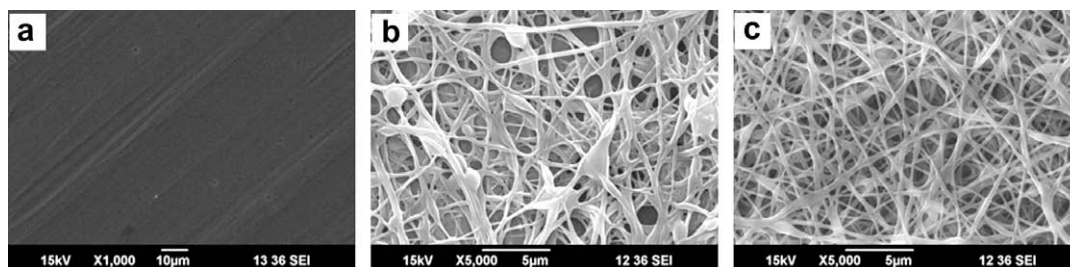


Fig. 5. SEM images of CMC/PVA fibrous scaffolds before and after cross linking. CMC/PVA nanofibers (control) in water for (a) 1 h and cross-linked CMC/PVA nanofibers in water for (b) 1 and (c) 48 h.

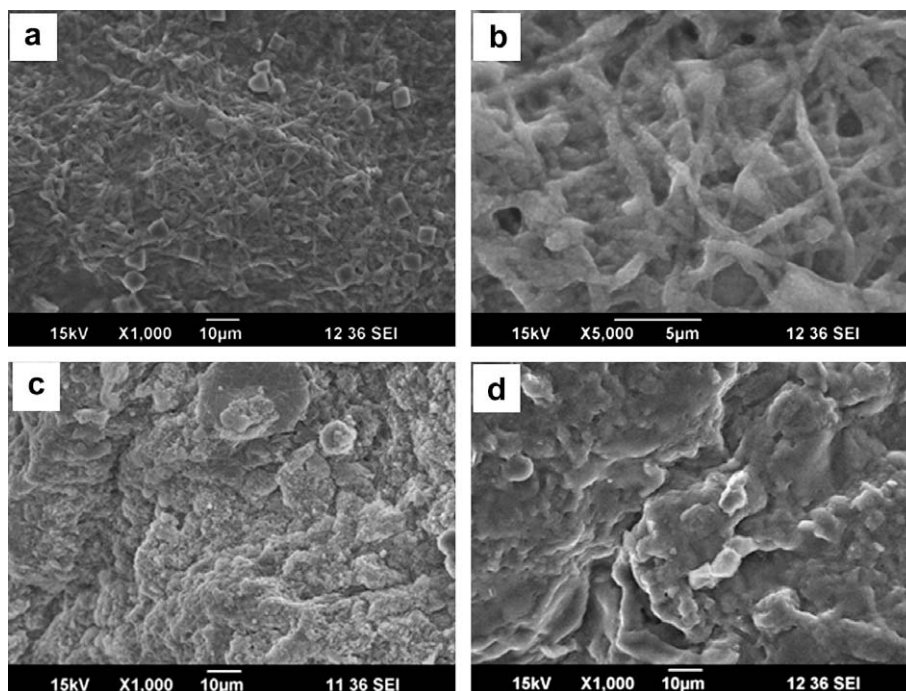


Fig. 6. SEM images of *in vitro* mineralized HAp on the surfaces of CMC/PVA scaffolds during different time intervals by alternate soaking method: (a) After 1 cycle (1000 \times) (b) after 1 cycle (5000 \times) (c) after 3 cycles and (d) after 5 cycles.

cycle. Upon time, after completion of 3rd and 5th cycles, all the fibrous structures were completely covered up with HAp (Fig. 6c and d). The material formed on the surface of the scaffold was found to be calcium phosphate as determined by the EDS spectra confirming the ability of formation of calcium phosphate formation on the surfaces of nanofibrous scaffolds. The ratio of Ca/P by EDS 1.89, which was slightly higher from the theoretical value 1.67, pointing out the formation of calcium rich HAp on surface of the scaffolds.

3.6. Cell adhesion and MTT assay

The level of toxicity of these fibrous scaffolds towards cell viability was evaluated using ISO10993-5 standard test method of indirect MTT assay. The extraction media were prepared from the fibrous scaffold either washed with PBS three times or unwashed. As described in method sections, hMSCs were grown in the presence of various concentrations of the extracts prepared from the washed and unwashed scaffolds. When cells were incubated with extracts obtained from washed scaffold, the viability of cells was not changed. This was similar to the negative control where cells were incubated only with the medium (Fig. 7). However, when cells were incubated with 100% extract obtained from unwashed

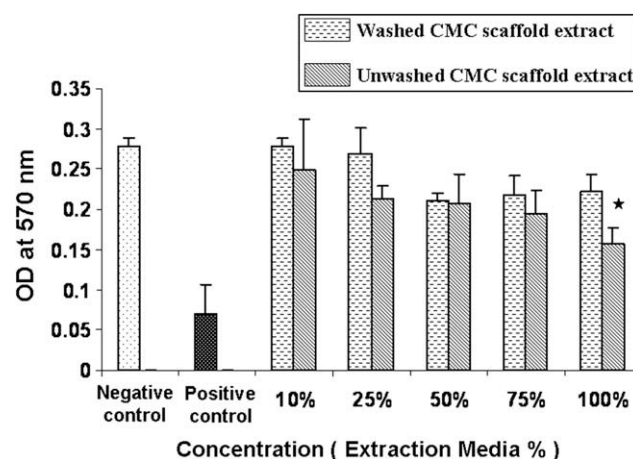


Fig. 7. Cytotoxicity effect of nanofibrous scaffolds. hMSCs were incubated with the supernatant obtained before and after washing the scaffolds. Negative control represents incubation of cells with the medium. Positive control represents incubation of cells with the 1% triton containing medium. *Significant difference compared to negative control; $P < 0.05$.

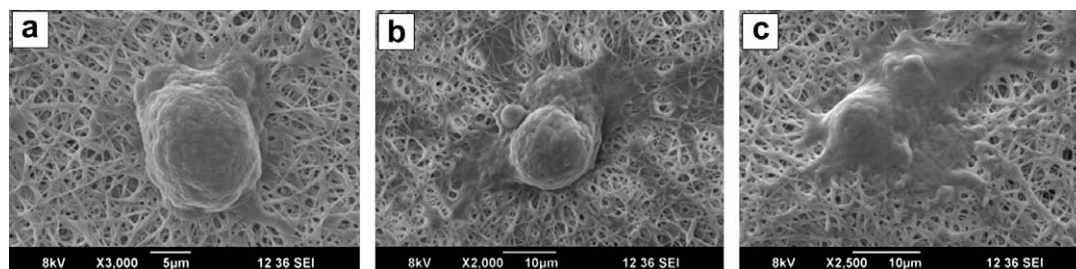


Fig. 8. SEM images of hMSCs attached on the surfaces of CMC/PVA scaffolds after (a) 12 h (b) 24 h and (c) 48 h of incubation.

scaffolds, there was a significant decrease on cell viability compared to the negative control. These results point out the possibility of the presence of glutaraldehyde traces as toxic in the scaffold after cross-linking.

3.7. Cell spreading

The utilization of the fibrous scaffolds for tissue engineering applications was further studied the SEM analysis. hMSCs were seeded on these scaffold and cell images at different time intervals were observed by SEM. Seeded hMSCs were adhered and spreaded well on the scaffolds in a time depended manner (Fig. 8). Attached cells remained in a more or less rounded morphology after 12 h of incubation and took a time period of 24 h to start spreading on the scaffolds (Fig. 8a and b). After 48 h, cells spreading became more prominent and cells became in a flat morphology on the scaffold surface (Fig. 8c). These results strongly suggest that the nanofibrous scaffolds reported here is suitable for tissue engineering applications.

4. Conclusions

CMC, a well-known biocompatible water-soluble polymer was successfully electrospun to nanofibers. PVA was selected as the polymer additive to produce electrospun nanofibers because of its good fiber forming, biocompatibility, and chemical resistance properties. The effect of viscosity and conductivity of the solution on fiber formation and the morphology of CMC/PVA bio-component fibers were studied. FTIR studies revealed the presence of CMC and PVA on the CMC/PVA scaffold. Since CMC and PVA are water-soluble polymers, insolubility of the scaffold in water was achieved by cross linking with glutaraldehyde. Mineralization studies showed that the material has the capability of forming calcium phosphate on the surface by alternate soaking technique. Human mesenchymal stem cells were able to adhere and proliferate on the fibrous scaffolds. Hence, these bioactive and biodegradable scaffolds may be suitable for tissue engineering applications.

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