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REVIEW ARTICLE

Bioactive Nano-Hydroxyapatite Doped Electrospun PVA-Chitosan Composite Nanofibers for Bone Tissue Engineering Applications

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Abstract | Combination of bioceramics with polymers to fabricate nanofibrous scaffolds holds enormous potential for bone tissue regeneration. In this study, we aim to incorporate HAp nanoparticles in trace doping amount in PVA-chitosan nanofiber matrix to fabricate PVA-chitosan composite nanofibers with improved performance for application as a bone tissue regeneration material. The diameter of the fabricated composite nanofibrous mat is estimated as 300 ± 121 nm. Beads free nanofibers mat with uniform morphology was ascertained for all sample groups by scanning electron microscopy (SEM) and the overall composition was assessed using Fourier transform infrared spectroscopy (FTIR) and energy dispersive X-ray spectroscopy (EDX). SEM images showed a homogeneous distribution of HAp nanoparticles in the composite nanofibers matrix. Further, X-ray diffraction (XRD) was performed to determine the crystallinity of the fabricated scaffolds. Swelling behavior and hydrolytic degradation of nanofibrous mats were subsequently evaluated by immersing in PBS buffer at pH 7.4 at physiological temperature (37 °C). The biocompatibility study of nanofiber scaffolds was performed with MC3T3 cells. Significantly higher cellular viability was observed on HAp nanoparticles incorporated composite nanofibrous scaffold surface after 7 days of culture in comparison to scaffolds without HAp.

Keywords: *Bone tissue engineering, Nanohydroxyapatite, Poly(vinyl alcohol), Chitosan, Composite nanofibers, Electrospinning, In vitro cytotoxicity*

1 Introduction

Bone defects and diseases have increased exponentially in the recent years making them one of the most expansive research areas in the tissue engineering. Available bone substitutes are not sufficiently adequate to meet clinical needs in terms of their functionality, degradation behavior, mechanical strength, and availability or cost while limitations of autologous or heterologous grafts persist[1–3]. Hence, new avenues of tissue engineering are explored with enthusiasm. Specifically, tissue engineered bone constructs have been posed to be advantageous due to their availability, biomimicry, low immunogenicity and limited secondary trauma in contrast to the clinical grafting process[4–6]. However, the complex, heterogeneous architecture and hierarchy of bone tissue makes fabrication of tissue-mimetic scaffolds challenging[7,8].

Several fabrication techniques such as freeze drying, phase separation, self assembly and electrospinning have been used for constructing three dimensional biomimetic scaffolds in which the cells are seeded and allowed to populate with secretion of their own extracellular matrix (ECM)[9]. They provide the initial template for regeneration of tissue and help in mimicking the original architecture of ECM[10]. Amongst several

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scaffold types, fibrillar nanostructures resemble natural ECM and provide favorable cell-material interaction. In this regard, electrospinning is one of the widely established fabrication strategies for development of ECM-mimicking nanofibrillar scaffolds[11]. Moreover, the technical simplicity and low tooling costs enhance the acceptability of this method. However, despite several functional advantages inadequate mechanical strength of polymeric nanofibrous scaffolds is still persisted as a key challenge for its widespread applications in load bearing tissue engineered implant applications[12]. Thus, several biological and structural requirements should be taken into consideration while selecting suitable biomaterial for fabrication of scaffolds for tissue engineering. Chitosan, a natural poly-β-(1–4)-D-glucosamine in a cationic form that is obtained by partial deacetylation of chitin, attracts a lot of attention due to its biocompatibility, biodegradability, antibacterial and antifungal properties[13]. Several attempts have been made conjugating chitosan with a variety of different biomaterials including calcium phosphate, hyaluronic acid, alginate or other therapeutic cytokines and biomolecules for potential applications in bone tissue engineering[14–18]. Despite such advantages, polycationic nature of chitosan makes it unsuitable for electrospinning. The main problem arises due to the repulsive forces between the polycations, preventing sufficient chain entanglement for forming nanofibers[13]. To overcome this problem, chitosan has been blended with poly (vinyl alcohol) (PVA) which is biodegradable, water soluble and has better nanofiber forming capability[19]. Further, it was suggested that blending with PVA can enhance the thermal stability and mechanical property of electrospun fiber membrane[20]. Several earlier reports have shown synthesis of PVA-chitosan electrospun nanofibers but the right proportion of PVA and chitosan is critical to obtain beadless and uniform fibers morphology[21–23]. It has been shown that with increasing PVA concentration from 10 to 90% in chitosan solution bead formation were reduced and fibers became thicker and more stable.

Here, it can be recalled that natural bone is formed by an "organic matrix-mediated" mineralization process in which collagen fibrils are self-assembled with highly ordered nanosize blade-like hydroxyapatite (HAp) crystal in 50/50 (v/v) or 80/20 (w/w) ratio[24]. Hence, tissue engineered scaffolds or bonegrafts fabricated with only polymer have inherent poor bioactivity and inferior osteoconduction. To address this limitation, several researchers have attempted to make three-dimensional (3D) nanocomposite scaffolds for bone tissue regeneration by reinforcing higher concentration of nano HAp particles into polymer matrix such as chitosan via porogen leaching method and lyophilization techniques[25–28]. For example, Kashiwazaki et al. fabricated HAp-chitosan porous nanocomposite scaffolds with 80/20 (w/w) blending ratio by porogen leaching method which exhibited good vascularity and bone regeneration capability in small animal model (rat)[25]. Kong et al. has showed that incorporation of nano HAp particles at a much lower concentration (12 wt%) to chitosan scaffold improved proliferation and alkaline phosphatase (ALP) protein expression of mouse osteoblast precursor cells (MC3T3-E1) suggesting potential use of nano-HAp/chitosan composite 3D scaffolds in bone tissue engineering[26]. However, those synthetic bone grafts with higher ceramic contents fail to achieve that structural organization of natural bone tissue and possess poor ductility due to absence of bone-like self-organizing interaction between the HAp and the extra cellular matrix components of the natural bone tissue[24]. Thus, the concentration of HAp phase in the composites have attracted diverse interests, with some studies laying emphasis on use of trace doping amounts of HAp that would facilitate desirable biodegradability and ductility to be better matched with those of natural bone. Consequently, Thein-Han et al. developed bone-like organic–inorganic biomimetic 3D nanocomposite scaffolds consisting of chitosan and varying trace amount of nano HAp particles (0.5, 1, and 2 wt%) for potential use as a bone tissue engineering material. Interestingly, significant increase in compression modulus as well as 1.5 times higher bone cell proliferation can be achieved with incorporation of only 1 wt% HAp nanoparticles into chitosan matrix in comparison to pure chitosan scaffold[27, 28]. However, most of these studies have been performed with freeze dried scaffolds, which do not mimic the nanofibrillar architecture of bone matrix.

In this study, we attempt to incorporate HAp nanoparticles in trace doping levels in PVA-chitosan nanofiber matrix to fabricate PVA-chitosan composite nanofibers with improved performance for application as a bone tissue regeneration material. Such polymer-ceramic composite scaffolds are expected to mimic the structure of natural bones by containing nano-sized HAp particles (at trace concentrations) along with organic nanofiber layers, which provides the site for osteoconduction and the polymeric matrix would facilitate desirable biodegradability and ductility to be better matched with those of natural bone.

To best of our knowledge only a few attempts have been made to incorporate nano HAp particles in trace doping amount in PVA-chitosan electrospun composite nanofibers mat[29, 30]. Amongst them, Celebi et al. reported fabrication of PVA-chitosan electrospun nanofibers consisting of Ag^{+}-incorporated HAp nanoparticles (0.1, 0.3, 0.5, and 1.0 wt% of nano HAp reinforcement) which exhibited antibacterial efficiency against *Escherichia coli*[30]. However, beaded fibers morphology and absence of any in vitro bone cell-scaffolds interaction study warrants further process optimization studies to obtain homogeneous and beads free fibrous mat. Thus, much effort has been invested in this work to optimize the blending concentration of polymers (i.e., PVA and chitosan) and ceramic (HAp) phases to produce homogeneous and beads free nanofibrous mat. For this purpose nano HAp was first ultrasonically suspended in aqueous solution and then mixed with the optimized blending ratios of PVA/chitosan spinning solution to fabricate beadless composite nanofibrous mat by conventional electrospinning technique. Physicochemical and structural characterization of the developed nanofibrous mats were further evaluated by XRD, FTIR, SEM, EDX and contact angle measurements. In vitro biocompability of composite nanofibrous mat was evaluated by culturing with mouse osteoblast cell line for up to 7 days culture period and compared with virgin PVA and PVA-chitosan electrospun nanofiber scaffolds.

2 Materials and Methods

2.1 Chemicals

Polyvinyl alcohol (PVA; 60–125 kDa), chitosan (3.8–20 kDa), poly (acrylic acid sodium salt) were procured from Himedia Laboratories Pvt. Ltd., India. Acetic acid glacial (CH_3COOH) was obtained from Fisher Scientific, USA. Diammonium hydrogen phosphate [$(NH_4)_2HPO_4$] was bought from Merck, USA. Calcium nitrate tetra-hydrate [$Ca(NO_3)_2.4H_2O$], di-ammonium hydrogen ortho- phosphate [$(NH_4)_2HPO_4$], triethanolamine (TEA) and ammonium hydroxide (NH_4OH) were procured from SD Fine-Chem, India. Ultra pure water (UPW) was used throughout all the processes and obtained using a Milli Q-System (Milli Q Academic Century, ZMQS50001, USA).

2.2 Preparation of Nano-Hydroxyapaptite (nHAp) Powder

The nano HAp powder was prepared in house by following a wet chemical method using analytical reagent grade $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH_4)_2HPO_4$ as precursors of calcium and phosphate ions, respectively as described in our earlier work[31]. Briefly, 100 mL of 1(M) solutions of $Ca(NO_3)_2{\cdot}4H_2O$ was taken into a 1 l beaker and stirred vigorously using a magnetic stirrer at 750 rpm in room temperature. Then, 100 mL of 0.6 (M) of TEA (capping agent) was added dropwise to the $Ca(NO_3)_2{\cdot}4H_2O$ solution at a rate of 3–5 mL/min under vigorous stirring condition. Following, 0.6 (M) of $(NH4)_2HPO_4$ solution was added dropwise into the beaker. The pH of the solution was always maintained at 10–12 by dropwise addition of ammonium hydroxide (NH_4OH) solution during mixing of TEA and $(NH4)_2HPO_4$ into $Ca(NO_3)_2{\cdot}4H_2O$ solution. The Ca:P molar ratio of $Ca(NO_3)_2{\cdot}4H_2O$ and $(NH4)_2HPO_4$ solution was kept as 1.67 in order to obtain stoichiometric nano HAp powder. Following this, the mixture was stirred for another 30 min at room temperature and then allowed to precipitate for 48 h. Finally, the gelatinous precipitate was collected and washed thoroughly by using UPW in a centrifuge (Heal Force Neofuge 18R, Shanghai Lishen Scientific Equipment Co. Ltd., China) at 5000 rpm with 5 min interval until the ammonia smell is completely removed. Afterwards, the white HAp precipitate was dried in the oven at 70 °C overnight, and subsequently ball milled (Retsch GmbH, Germany) for 24 h in UPW medium in which certain amount of poly (acrylic acid sodium salt) was added as deflocculating agent. After ball milling, HAp powder was dried in the oven at 70 °C overnight to obtain nanosized HAp powder. Finally, the dried nano HAp powder was ground in an agate mortar pestle to break any agglomeration and subsequently used in physicochemical characterizations and fabrication of HAp incorporated PVA-chitosan nanofibers by electrospinning technique.

2.3 Fabrication of PVA, PVA-CHT and HAp Incorporated PVA-CHT Composite Nanofibers Mat

Pre-blending concentration of PVA solution (i.e., 15% (w/v) PVA solution in ultra-pure water) and chitosan solution (i.e., 1% (w/v) chitosan solution in 1% (v/v) acetic acid) is being selected based on previous studies and process optimizations in order to produce homogeneous and beads free nanofibrous mat[22, 23]. At first, 15% (w/v) PVA solution was prepared by mixing PVA in UPW at 70 °C for 6 h using a magnetic stirrer at 600 rpm. Then, 1% (w/v) chitosan solution was made in 1% (v/v) acetic acid in UPW and

kept under constant stirring at 600 rpm for 24 h to obtain a clear solution. In our preliminarily studies, two different PVA/chitosan blend ratio i.e., 80:20 and 90:10 (v/v) have been explored, and fibrous mat quality was found superior at PVA-chitosan blending ratio 90:10 (v/v) under optimized spinning conditions. Consequently, we have selected PVA/chitosan (PVA-CHT) blending ratio 90:10 (v/v) as the final blend in this study and PVA-CHT solution was prepared by mixing the appropriate volume of stock 15% (w/v) PVA solution and 1% (w/v) chitosan solution and stirred for 30 min at 600 rpm in room temperature to achieve homogenous mixture.

In this study, we aimed at fabrication of composite electrospun nanofibrous mat by incorporating bioactive HAp nanoparticles in a trace doping amount into PVA-chitosan matrix and compared it's microstructural, physicochemical as well as in vitro bone cell-materials interactions with PVA and PVA-chitosan nanofibers pertinent to bone tissue engineering applications. Thus in our preliminary studies, two different concentration of nano HAp (0.5 and 1.0 wt%) were investigated for incorporation into PVA/Chitosan (90:10 v/v) nanofibrous mat based on previous studies and found 0.5 wt% HAp reinforcement as optimal composition in view of beadless fibers morphology and bone cell proliferation[29,30]. Consequently, a fixed concentration (i.e., 0.5 wt%) of nano HAp powder was selected to incorporate into the PVA-CHT polymer matrix for fabrication of HAp/PVA-CHT composite fibers and subsequent physicochemical and in vitro biological characterizations. For that purpose, nano HAP powder (nHAp) was first suspended in aqueous solution (UPW) supplemented with minute quantity of deflocculating agent ((acrylic acid sodium salt) and thoroughly mixed at 300 rpm for 1 h followed by the ultrasonication for 10 min. Then, desired volume of ultrasonicated HAp stock solution was mixed with blended polymer solution (PVA-CHT) to prepare nano HAp/PVA-CHT spinning solution (0.5 wt% HAp, w/v) and vigorously stirred for 2 h to achieve homogeneous distribution of nano HAp powder in polymer blend solution.

The PVA, PVA-CHT and 0.5 wt% nano HAp incorporated PVA-CHT (0.5. HAp/PVA-CHT) composite nanofibers were fabricated by electrospinning technique (E-SPIN Nanotech Pvt. Ltd., IIT Kanpur, India). 10 mL each group of solution was filled into a 5 mL syringe with a stainless steel needle for electrospinning. Then, the electrospinning process was carried out at room temperature with a constant feeding rate of 1 mL/h and the distance between the needle tip and collector was kept at 14 cm. The aluminum foil was used as the collector for the nanofibers. The applied voltage was varied between 20 and 25 kV to obtain beadless and uniform nanofibers mat. Extensive preliminary studies were carried out to optimize the electrospinning parameters in order to obtain reproducible, uniform and beadless nanofibers. At the end of spinning process, each group of the fibrous mat was collected from the aluminium foil, dried and stored in a vacuum dessicator for further characterizations.

2.4 Characterization of the Samples

X-ray diffraction (XRD) pattern of as prepared nano HAp powder and electrospun nanofiber mats was determined by a X-ray Diffractometer (X'Pert Pro MPD Diffractometer, Panalytical, Alnelo, Netherlands) with scanning range 0°–60° to assess different crystalline phases and structures. Fourier Transform Infrared (FTIR) spectroscopy (IRAffinity-1S, IRAffinity-1, MIRacle 10, SHIMADZU, Japan) was carried out for analying the molecular fingerprints of different test samples. The surface morphologies and elemental composition analysis of nano HAP powder and electrospun fibrous mats were studied by Scanning electron microscopy (SEM) and Energy dispersive X-ray (EDX) analyses (Phenom ProX, Phenom World B.V., Netherlands). Surface properties for different sample groups was assesed by measuring the static contact angles of sessile drops of DI water using a face contact angle setup equipped with a camera (Surface tensiometer surfectens 4.5, OEG GmbH, Germany). For this purpose, 2 mL polymer blend solution from each of sample group (PVA, PVA-CHT, and 0.5HAp/PVA-CHT) was taken in a 5 mL syringe and drop casted into film on a glass slide and subsequently air dried prior contact angle measurment. Minimum three measurement was carried out on triplicate samples for each group and the average contact angle values were calculated.

2.5 Swelling Behavior of PVA, PVA-CHT and 0.5HAp/PVA-CHT Nanofibrous Mat

Swelling behavior of PVA, PVA-CHT, and 0.50HAp/PVA-CHT nanofibrous mats was assessed by immersing in PBS buffer at pH 7.4 at physiological temperature (37 °C). Briefly, nanofibrous mats were cut into 20×20 mm^2 test specimen and each sample was immersed into 50 mL phosphate buffer solution (pH 7.4) at 37 °C in an incubator up to 16 h (960 min). Prior immersion,

initial dry weight (W_d) of each test specimen was measured. After specific time intervals, nanofibrous mats were taken out of PBS buffer and carefully removed the surface water by holding over tissue paper for a few seconds. Subsequently, the swell weight (W_s) of each test specimen was measured. The experiment was done in triplicates for each scaffold. The swelling ratio (%) was calculated using Eq. 1 by gravimetric method[32].

$$\text{Swelling ratio (\%)} = \frac{W_s - W_d}{W_d} \times 100 \quad (1)$$

where, W_d stands for initial dry weight and W_s refers to swell weight of the nanofibrous mat at respective time point.

2.6 Hydrolytic Degradation of PVA, PVA-CHT and 0.5HAp/PVA-CHT Nanofibrous Mat

Hydrolytic degradation of PVA, PVA-CHT, and 0.50HAp/PVA-CHT nanofibrous mats was evaluated by immersing in PBS buffer at pH 7.4 at physiological temperature (37 °C) for 1, 4 and 7 days time point. Briefly, nanofibrous mats were cut into 20×20 mm^2 pieces for each sample group and dry weight of each test specimen was measured (W_o). Following this, each sample was immersed in 50 mL phosphate buffer solution (pH 7.4) and kept in an incubator at 37 °C. After specific time intervals, samples were taken out of PBS buffer and dried in an oven at 55 °C. Subsequently, dried weight (W_t) of each test sample was measured and the weight loss (%) was calculated using Eq. 2 by gravimetric method[33]. The experiment was done in triplicates for each scaffold.

$$\text{Weight loss (\%)} = \frac{W_o - W_t}{W_o} \times 100 \quad (2)$$

where, W_o stands for initial weight and W_t refers to dry weight of the nanofibrous mat at respective time point.

2.7 In vitro Bone Cell–Material Interactions

In vitro biocompability of different electrospun nanofiber mats (PVA, PVA-CHT and 0.5HAp/PVA-CHT) was evaluated using mouse osteoblast cell line (MC3T3-E1, ATCC, USA). All three groups of nanofibrous mats were cut into small test specimen (10 mm × 10 mm) and sterilized by immersing in 70% EtOH for 30 min, followed by rinsing with sterile UPW for 15 min and ultraviolet (UV) exposure for 1 h in aseptic condition. The MC3T3 cells as used in the study were prepared by culturing in α-MEM growth medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic solution in a T75 tissue culture flask (Himedia Laboratories Pvt. Ltd., India). The cultured cells were kept in the incubator (Cel-Culture, ESCO) at 37 °C with 5% CO_2 and 95% humidity and the culture medium was replaced with fresh medium at every 2–3 days. Following this, the confluent cells were trypsinized (trypsin/EDTA; Invitrogen Corporation, USA), and cell suspensions were prepared. Finally, cells were seeded on the surface of nanofiber mats at a density of 10,000 cells/sample placed in a 24-well cell culture plate by adding 100 µL of cell suspension to each sample and incubated for 4 h in the incubator at 37 °C for cell adherence. After that, 1 mL α-MEM growth medium supplemented with 10% FBS and 1% antibiotic–antimycotic was added to each well. Cultures were maintained up to 7 days in the incubator at 37 °C under an atmosphere of 5% CO_2 and 95% humidity and culture media were changed every 2 days. Samples were then collected after specific time interval to study the cell proliferations and morphology.

2.7.1 Cell Proliferation Using the MTT Assay

In vitro cytotoxicity of different nanofibrous mats was quantitatively assessed and compared by measuring the viable cell proliferation on different sample surfaces after 1, 3 and 7 days culture timepoint using the MTT assay kit (Sigma Aldrich, USA). Briefly, the MTT reagent [3-(4, 5dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] was dissolved in phosphate buffer solution (PBS, Gibco) at a concentration of 5 mg/mL. After the specified incubation timepoint, freshly prepared MTT solution was mixed with culture media in a volume ratio of 1:9. After mixing, 1 mL of solution was added to each sample. The mitochondrial succinate dehydrogenase reduced MTT to a dark purple formazan product. After 3 h incubation, the formazan crystals was saturated in 500 µl of DMSO (Dimethyl sulfoxide, Sigma) solution. An aliquot of 100 µl was transferred into a 96-well plate and optical density was measured using a plate reader (iMark, Bio-Rad) at 595 nm. Triplicate samples ($n = 3$) were used in MTT assay to ensure reproducibility. Cell proliferation (%) was calculated by normalizing the measured optical densitiy of each sample group to measured optical density of PVA for that particular culture time point (data not shown).

2.7.2 Cell Morphology by Fluorescence Imaging

Cell morphology was qualitatively analyzed after 7 days culture period by staining the cell nuclei with a flourescent dye DAPI (4',6-diamidino-2-phenylindole Catalogue No: D1306, Thermo Fisher) and cunterstaining the cell membrane and cytoplasmic proteins with another flourescent dye FITC (Fluorescein isothiocyanate isomer I, Thermo Fisher) followed by fluorescence imaging. Briefly, after 7 days incubation period the samples ($n=2$, duplicate condition) cultured with MC3T3-E1 cells were rinsed with 1X PBS for 3 times/5 min each, fixed in 3.7% formaldehyde for 10 min. This was followed by 3 times wash in PBS. Later the cells were permeabilized by immersion in 0.2% Triton X-100 for 5 min. Triton X was aspirated followed by washing with PBS. Then FITC staining solution (2.5 µg/mL) was added to each sample well and kept for 5 min and then washed with PBS. Lastly, the cells were imaged ($\lambda_{ex} \sim 359$ nm, $\lambda_{em} \sim 461$ nm when FITC is bound to cytoplasm) under reflected light fluorescence microscope (OlympUSBX51TRF, Tokyo, Japan).

2.8 Statistical Analysis

Data for contact angle and MTT assay are presented as mean ± standard deviation. Statistical analysis was performed on MTT assay results using student's t test and P value < 0.05 was considered significant.

3 Results and Discussion

3.1 SEM Morphology and EDX Elemental Analyses

SEM morphology of as synthesized nano HAp powders (nHAP) and representative elemental analysis (EDX analysis) results are presented in Fig. 1a, b, respectively, Evidently, granular HAp nanoparticles with an average particle size below 100 nm was obtained by wet chemical precipitation method (Fig. 1a). Presence of some agglomerates can also be seen due to high surface energy of the nano-sized HAp particles. EDX spectrum results acquired from the HAp particles further confirmed the presence of Ca, P and O elements indicating the HAp chemical composition (as shown in Fig. 1b). Morphologies of electrospun virgin PVA, PVA-CHT blend and 0.5HAp/PVA-CHT composite nanofibrous mats are presented in Fig. 1c–e, respectively. A beads-free continuous and nonwoven structure with interconnected porous morphology can be seen for all the electrospun nanofibrous mats irrespective of their composition. Pure or virgin PVA nanofibers were smooth and homogeneous but bimodal in nature with an average diameter of 440 ± 196 nm (Fig. 1c). In case of PVA-chitosan blend (PVA-CHT) nanofibers were more uniform with an average diameter of 395 ± 162 nm as it is believed that the functional groups of chitosan formed hydrogen bonds with PVA to give more stability and defect-free nanofibers morphology (Fig. 1d). In case of composite nanofibers (0.5HAp/PVA-CHT) successful incorporation of HAp nanoparticles was seen in the form of aggregated mass inside the PVA-CHT blend polymer nanofibers matrix. Average nanofibers diameter was reduced to 300 ± 121 nm for composite nanofibers (0.5HAp/PVA-CHT) with the incorporation of HAp nanoparticles (Fig. 1e) and the surface of fibers was rougher than that of pure polymer fibers due to the inclusion of HAp nanoparticles. It can be recalled that a rough nanofiber surface produced by the embedded HAp nanoparticles is expected to increase surface wettability and promote bone cell attachment and osteogenic differentiation[34]. Incorporation of the HAp nanoparticles can be confirmed from the associated elemental analysis results (EDX) acquired from the embedded particles into the polymer nanofibers matrix (as shown in Fig. 1f).

3.2 FTIR Analysis

The FTIR spectra of nano HAp powder and electrospun PVA, PVA-CHT and 0.5HAp/PVA-CHT nanofibers are presented in Fig. 2a. In case of nano HAp powder, broad peaks at 3450 cm^{-1} and 1644 cm^{-1} are mainly due to adsorbed water[35]. Further, phosphate stretching mode, phosphate bending mode were seen at 1034 cm^{-1} and 605 cm^{-1} respectively[36, 37]. Besides, C–O stretching vibration of CO_3^{2-} were found at 1454 cm^{-1} and 1414 cm^{-1}, respectively. These characteristic peaks suggested successful synthesis of HAp. In pure PVA nanofibers FTIR spectra, two peaks were obtained at 3300 cm^{-1} and 1640 cm^{-1} for O–H stretching vibration and bending vibration, respectively. The FTIR spectra of PVA-CHT nanofibers revealed the band at 1430 cm^{-1} due to amide bond (amide-II) of chitosan that did not appear in the pure PVA spectra[38]. In 0.5HAp/PVA-CHT composite nanofibers, besides the PVA-CHT characteristics peaks at 3300 cm^{-1} is due to –OH stretching vibration. Besides, the amide bond (amide-II) of CHT and C–O stretching vibration peak of CO_3^{2-} of nHAp were merged and was found at 1423 cm^{-1} with high intensity. Additional peaks were observed at 1096 cm^{-1} and 615 cm^{-1}. These additional bands

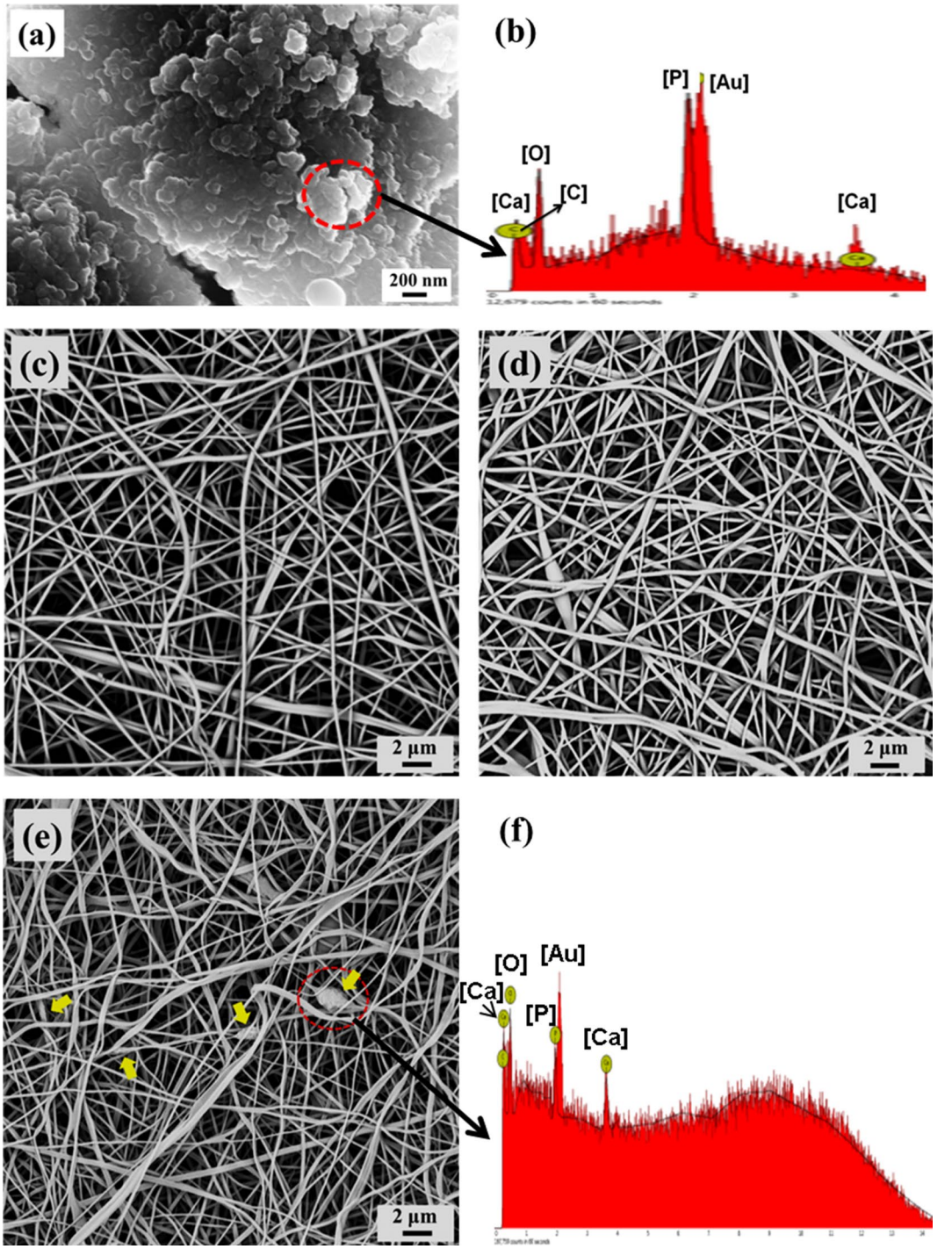

Figure 1: SEM micrographs illustrating the powder morphology of **a** as synthesized nano HAp (nHAP) dried at 70 °C for overnight, and surface morphology of electrospun **c** PVA, **d** PVA-CHT and **e** 0.5HAp/PVA-CHT nanofibers. EDX elemental analysis results showing chemical composition of **b** nano HAp powder (nHAP) and **f** 0.50HAp/PVA-CHT composite nanofibers. (small arrows are indicating the incorporated HAp nanoparticles into nanofibers matrix).

are assigned to the characteristic peaks of HAp confirming the presence of HAp nanoparticles into composite nanofibers. Overall, FTIR analyses results suggest the plausible incorporation of HAp nanoparticles into the polymer matrix for successful fabrication of 0.5HAp/PVA-CHT composite nanofibers.

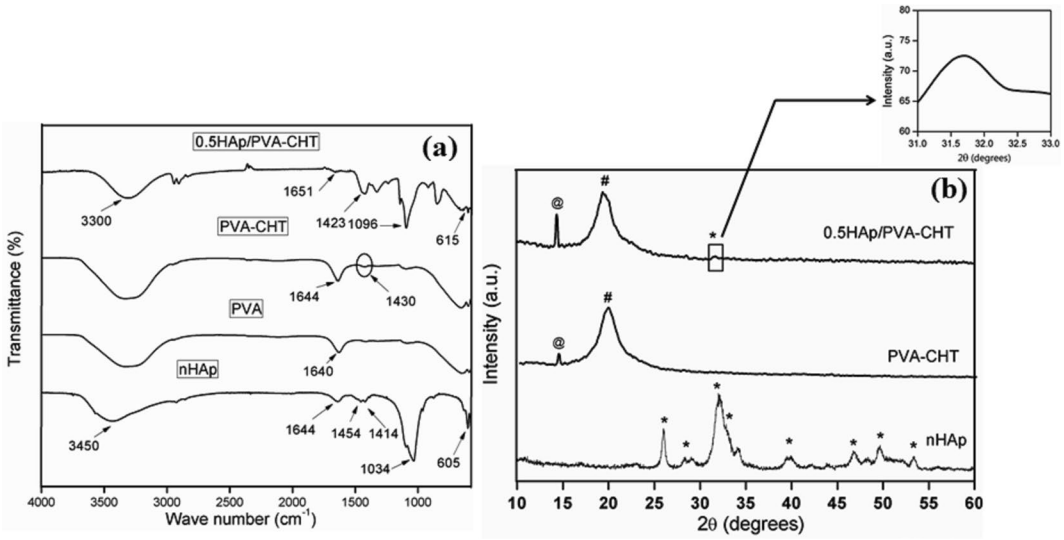

Figure 2: **a** FTIR spectra of nano HAp powder (nHAP) and electrospun PVA, PVA-CHT, 0.5HAp/PVA-CHT nanofibers, **b** XRD patterns of nano HAp powder (nHAP) and electrospun PVA, PVA-CHT, 0.5HAp/PVA-CHT nanofibers. The inset shows the XRD peak corresponding to the HAp phase for 0.5HAp/PVA-CHT composite nanofibers (*Peak corresponding to HAp phase, #Overlapping peaks corresponding to both PVA and chitosan, @Artifact from sellotape that was used for fixing the nanofiber mat in sample holder during XRD analysis).

3.3 XRD Analysis

The phase of the HAp powders and fabricated electrospun nanofibrous mats was confirmed by X-ray diffraction analysis. Figure 2b shows the XRD patterns for as synthesized nano HAp powder dried at 70 °C along with PVA-chitosan and HAp incorporated PVA-chitosan composite nanofibers. Evidently, the four major peaks in as-synthesized HAp powder XRD pattern at 31.8°, 32.54°, 26.12° and 34.23° are the diffraction peaks from (211), (112), (002) and (202) planes and matching well with the JCPDD PDF No. 9-432[39]. From this observation it can be inferred that phase pure HAp power was prepared under the present experimental conditions. In addition, extensive peak broadening and overlapping of the diffraction reflections, as in the case of natural bone, suggesting nano sized and low crystallinity of the HAp particles[40]. In case of PVA-chitosan nanofibers, (PVA-CHT) the broad peak at about 20° is assigned to the overlapping peaks of both PVA and chitosan and was indicative of successful blending of PVA onto the backbone of chitosan moiety[41]. The XRD pattern of HAp incorporated PVA-CHT composite nanofibers shows the presence of broad (211) diffraction peak of between 31° and 32° confirming the presence of HAp phase along with characteristic peak of PVA-chitosan blend at 20°. This decrease in HAp crystallinity as evident from broader HAp peak may be caused by the presence of the chitosan matrix and trace amount of HAp content (0.5 wt%, w/v) in the composite nanofibers. A similar observation was also reported by Wang et al.[42]. Overall, XRD analyses results are also in good agreement with SEM, EDX and FTIR observation HAp nanoparticles are being successfully incorporated into the PVA-chitosan nanofiber matrix.

3.4 Contact Angle Measurement

Wettability of different samples was assessed by measuring the static contact angles of sessile drops of DI water on PVA, PVA-CHT, 0.5HAp/PVA-CHT drop casted films. The films were prepared from each of spinning solutions and used as specimen instead of nanofibrous mats which were not suitable for accurate measurement due to their inherent porous structure. The comparative plot of water contact angle as measured for PVA, PVA-CHT and 0.5HAp/PVA-CHT samples are presented in Fig. 3a. Overall, all the sample groups are found to be hydrophilic in nature which is beneficial for bone cell attachment and proliferations. The average contact angle of pure PVA was found to be at $18.9° \pm 0.78°$ whereas PVA-CHT exhibited increased wettability or reduced water contact angle of $16.63° \pm 0.25°$ due to the availability of –OH group in the saccharides moieties in chitosan[43]. Interestingly, 0.5HAp/PVA-CHT composite exhibited maximum wettability amongst all samples with a

lowest contact angle value of 12.53° ± 0.25°. It is believed that increased surface roughness due to the exposure of HAp nanoparticles might have helped to increase its hydrophilicity[44]. Digital pictures of water contact angles as measured for different samples are in good agreement clearly showed more spreading of water droplet on HAp incorporated PVA-CHT composite surface in comparison to pure PVA and PVA-CHT samples.

3.5 Swelling Behavior of PVA, PVA-CHT and 0.5HAp/PVA-CHT Nanofibrous Mat

The swelling behavior of the nanofibrous mat plays an important role in cellular adhesion and proliferation as it influences the fluid intake capacity of the scaffold. Figure 3b shows the swelling behavior of PVA, PVA-CHT and 0.5HAp/PVA-CHT nanofibrous mat after immersing in PBS buffer up to 960 min (16 h) at pH 7.4 at physiological temperature (37 °C). It can be noted that the increase in weight after immersion in PBS buffer indicates the swelling behavior of the nanofibers and the swelling ratio (%) was calculated by normalizing the increased weight for swelling to initial dry weight of nanofibrous mat. The degree of swelling was evidently increased for PVA-CHT in comparison to PVA, while 0.50HAp/PVA-CHT sample showed maximum swelling amongst all groups. Interestingly, maximum swell ratio (%) of 130.18%, 142.42% and 194.02% could be achieved for PVA, PVA-CHT, and 0.50HAp/PVA-CHT, respectively after 810 min (13.5 h) immersion period. It is believed that incorporation of chitosan leads to increase in hydrophilicity of PVA-CHT nanofibers due to the presence of functional amino and hydroxyl groups in chitosan[45]. Additionally, the incorporation of nano HAp into composite nanofibers has further increased the hydrophilic nature of the scaffolds due to presence of hydroxyl groups in HAp group that increases the buffer solution intake. This observation is in good agreement with contact angle results which has markedly confirmed that 0.5HAp/PVA-CHT composite possesses maximum wettability amongst all samples (Fig. 3a). However, the swelling ratio (%) was found to be decreased after 810 min (13.5 h) indicating the degradation and leaching of polymers by dissolution that resulted in the decrease in swelling ratio[45]. Similar results were also reported by other researchers when HAp was incorporated into chitosan/PLA blend and BisGMA[46, 47].

3.6 Hydrolytic Degradation of PVA, PVA-CHT and 0.5HAp/PVA-CHT Nanofibrous Mat

Hydrolytic degradation is an important useful process that influences scaffolds biodegradability and strength to support cell proliferation and new tissue formation. The hydrolytic degradation behavior of PVA, PVA-CHT and 0.5HAp/PVA-CHT nanofibers after immersing in PBS buffer up to 7 days at pH 7.4 at physiological temperature (37 °C) is shown in Fig. 3c. Decrease in scaffold weight after immersion in PBS buffer specifies the hydrolytic degradation behavior of the nanofibers and weight loss (%) was calculated by gravimetric method. From Fig. 3c it can be observed that after day 1 the % weight loss was 5.9%, 8.30%,

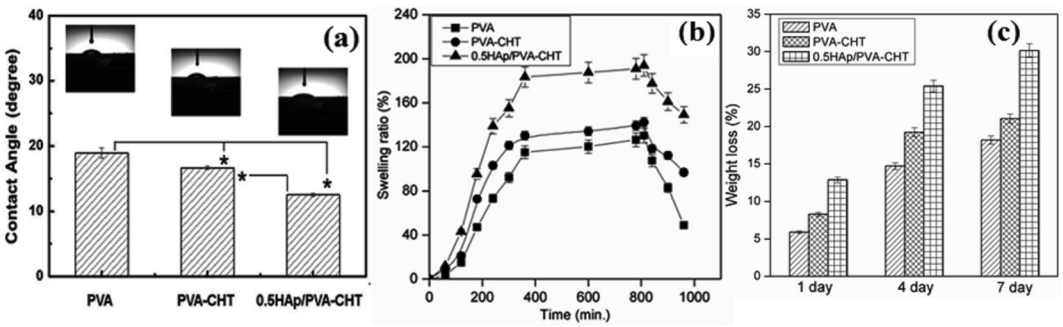

Figure 3: **a** Water contact angles as measured on PVA, PVA-CHT, 0.5HAp/PVA-CHT drop casted films. Digital pictures of water contact angles as measured for different samples are presented in the insets. Statistical analysis indicates that the differences in contact angles among various samples are significant (*$P<0.05$, $n=9$), **b** swelling ratio of PVA, PVA-CHT and 0.5HAp/PVA-CHT nanofibrous mat after immersing in PBS buffer up to 960 min (16 h) at pH 7.4 at physiological temperature (37 °C), **c** hydrolytic degradation behavior of PVA, PVA-CHT and 0.5HAp/PVA-CHT nanofibers after immersing in PBS buffer up to 7 days at pH 7.4 at physiological temperature (37 °C).

and 12.90% for PVA, PVA-CHT, and 0.50HAp/PVA-CHT scaffolds, respectively. It is believed that the increase in weight loss for HAp incorporated nanofibrous scaffolds over PVA-CHT and PVA is because of higher scaffold hydrophilicity which leads to higher buffer solution uptake that promotes hydrolysis. Similarly, incorporation of chitosan into PVA moiety for PVA-CHT scaffold increases the hydrophilic nature of the nanofibers that promotes degradation. Further, higher buffer solution uptake also accelerates the erosion and diffusion of chitosan. This rapid degradation process during the initial period is matching well with wettability and swelling behavior of different scaffolds as presented in Fig. 3a, c, respectively. From Fig. 3c it can be clearly seen that the rate of degradation is reduced with increasing the immersion time period as the % weight loss was found 14.40% and 18.18% (PVA), 19.23% and 21.05% (PVA-CHT), 25.39% and 30.15% (0.5HAp/PVA-CHT) at day 4 and day 7, respectively. This decrease in degradation rate with increasing immersion period can be attributed to the stabilization in hydrolysis kinetics and lower buffer solution intake mechanism. Overall, the observed hydrolytic degradation behavior of all nanofibrous scaffolds is typical of other biodegradable natural polymers[48, 49] Similar results were also reported for other HAp incorporated polymer nanocomposite scaffolds[50, 51]. The effect of chitosan and HAp nanoparticles in degradation behavior of scaffolds is an important finding and scaffolds degradation rate can be potentially controlled by controlling their compositions for patient specific applications. In addition, scaffolds stability can also be improved by using suitable cross-linkers[52].

3.7 In Vitro Bone Cell–Material Interactions

Cellular viability on different scaffold composition was assessed through the MTT assay (Fig. 4a). Viability of osteoblast cells markedly increased after 3 days of culture in all study groups in comparison to that of 1 day culture. However, no significant difference in cell viability was observed among all three study groups at 3 days culture period. Interestingly, after 7 days of culture the viability and proliferation of osteoblast was significantly increased in 0.5HAp/PVA-CHT nanofibers in comparison to PVA-CHT nanofibers and control pure PVA nanofibers groups. It has been observed that 0.5HAp/PVA-CHT fibers showed a maximum 2.76 fold increase in cell proliferation in comparison to PVA nanofibers after 7 days of culturing. On the other hand, PVA/CHT nanofibers also showed a moderate 1.40 fold increase in cell proliferation with respect to PVA fibers at the same time point. Most importantly, doping of nano HAp showed a 1.98 fold increase in bone cell proliferation when comparison is made between 0.5HAp/PVA-CHT and PVA-CHT nanofibers after 7 days culture period. Such increasing trend in cellular viability in nHAp composite scaffolds indicate the possibility of release of Ca^{2+} and PO_4^{3-} ions from the construct could potentially create conducive environment for better bone cell proliferation over other polymeric scaffolds groups. In addition, hydrophilic surfaces of composite nanofibrous scaffolds are expected to favor cell adhesion as well as enhance ionic interactions between cell and fibers leading to accelerated cell proliferation. Further, qualitative FITC fluorescence cell morphology results (as shown in Fig. 4b) indicate maximum cellular adhesion and proliferation in 0.5HAp/PVA-CHT nanofibrous mat surface at 7th day of culture in comparison to other sample groups. Evidently, this observation is in good agreement with quantitative MTT

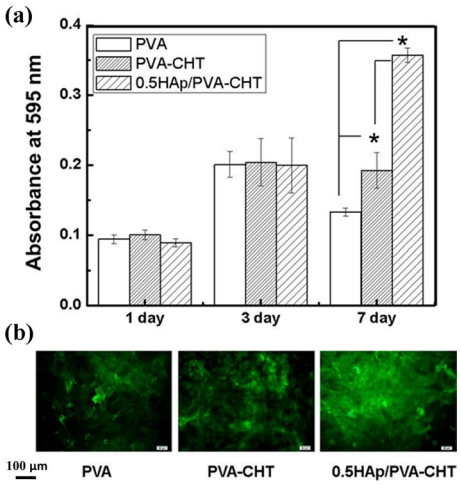

Figure 4: **a** Optical density measurement illustrating mouse osteoblast (MC3T3-E1) cell proliferation on different nanofibrous mat surfaces after 1, 3, and 7 days culture period. Statistical analysis indicate that the differences in cell densities among various samples are significant at 7 days timepoint (*$P<0.05$, $n=3$), **b** Fluorescence micrographs showing morphology of MC3T3-E1 cells stained with FITC on electrospun PVA, PVA-CHT, 0.5HAp/PVA-CHT nanofibrous mat surfaces after 7 days culture timepoint.

assay results. In support of beneficial effects on the developed composite fibers in bone tissue engineering, researchers have also reported that incorporation of doping concentration (5 wt%) of nano HAp particles in hydrogel matrices result in increased extracellular matrix production by stem cells reflected by increase in both eleastic modulus as well as formation of extra cellular matrix components[53].

4 Conclusions

In present study, HAp nanoparticles doped electrospun PVA-chitosan composite nanofibrous mat was successfully fabricated with improved performance for potential application as a bone tissue regeneration material. The SEM and EDX images showed successful encapsulation of HAp nanoparticles into the composite nanofibrous scaffolds. FTIR spectra exhibited the molecular fingerprints of composite scaffolds and further confirmed the successful fabrication of HAp incorporated polymer scaffolds. Besides, incorporation of nano HAp improved the hydrophilicity as well as swelling behavior of composite scaffolds and significantly enhanced the proliferation of osteoblast cells. Further, the rate of in vitro hydrolytic degradation was also found to be decreased for all sample groups with increasing immersion period. Maximum viability and good surface coverage of MC3T3 cells on composite scaffold surfaces have been observed after 7 days of culture. Overall the study suggests the incorporation of HAp nanoparticles improves the biocompatibility as well as bioactivity of PVA-chitosan composite scaffolds for osteoblast and such composite scaffolds may serve as a good template for bone tissue engineering.

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