

# Electrospun fibrous web of collagen–apatite precipitated nanocomposite for bone regeneration

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**Abstract** Electrospinning is regarded as a facile tool to generate biomaterials into a nanofibrous structure. Herein a nanofibrous web constituted of collagen and hydroxyapatite (HA) was produced from their co-precipitated nanocomposite solution by using the electrospinning method. The co-precipitated sol was freeze-dried and the dried product was dissolved in an organic solvent for the electrospinning. The electrospun web showed a well-developed nanofibrous structure with HA contents of up to 20 wt%. The internal structure of the collagen–20 wt%HA nanofiber revealed highly elongated apatite nanocrystallines precipitated within the collagen matrix. However, above the HA content of 30 wt% the nanofibrous structure could not be preserved due to the formation of beads. The MC3T3-E1 osteoblastic cells were shown to adhere and grow actively on the collagen–HA nanofibrous web. The alkaline phosphatase (ALP) activity expressed by the cells on the collagen–20 wt%HA nanofiber was lower at day 7, but was higher at day 14 than that on the pure collagen nanofiber. Based on the study, the newly-developed collagen–HA nanofiber may be useful as a cell supporting substrate in bone regeneration area.

## 1 Introduction

For the successful reconstruction of skeletal defects, a range of materials including ceramics, polymers and their composites have been developed, and some have shown a level of promising clinical outcome [1–3]. The collagen–hydroxyapatite (HA) nanocomposite has been regarded as a promising choice of material in the bone regeneration field [4]. The extracellular matrix of bone, basically constituted of hydroxyapatite (HA) nanocrystallines and collagen fibrils, is a typical inorganic–organic nanocomposite [5]. The collagen–HA nanocomposite system has been developed into forms of lyophilized powder, pressed bulk, porous foam and thin membrane for the specific applications [6–9]. The bone-mimicking characteristics of the collagen–HA products mainly attribute to the good bone cell responses *in vitro* and bone formation *in vivo*. In particular, the organization level of the collagen and HA has been shown to significantly affect the physical and biological properties of the nanocomposites.

Due to the recent advances in nanotechnology, it was possible to develop many types of biomedical materials with structure and organization on the nanoscale. The electrospinning technique allowed medical materials to be generated into a nanofibrous structure [10, 11]. Polymeric materials either in natural- (collagen, chitosan, and hyaluronic acid, etc.) or synthetic-base (polylactic acid: PLA, polyglycolic acid: PGA, polycaprolactone: PCL, and their copolymers, etc.) have been fabricated into nanofibers for the regeneration of tissues [12–16]. Because of their morphological trait, the nanofibrous webs have often shown better cellular responses such as initial adhesion and further matrix synthesis, in comparison with their dense counterparts.

For the regeneration of bone, the nanofibers with polymeric compositions such as collagen, chitosan, PLA, and

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PCL, have firstly been studied and results have shown their good cellular adhesion and growth and thus their possible use as bone cell supporting matrices [13, 14]. However, the composition of the polymeric nanofibers should further be improved in order to better trigger initial cell interactions and to stimulate a series of osteogenic processes, including bone cell differentiation and bone matrix synthesis.

Recent studies on the nanofibers for bone regeneration have been directed at producing polymeric composites with bioactive inorganics, which include PCL with CaCO<sub>3</sub>, PLA with HA, and gelatin with HA [17–19]. When compared to the polymeric individuals, their composites with bioactive inorganics have shown better *in vitro* osteogenic properties, such as the expression of alkaline phosphatase and osteocalcin. However, one of the significant challenges considered in the production of the composite nanofibers by the electrospinning is that the inorganic components are often not well distributed within nor properly organized with the polymers, hampering the formulation of the composite into nanoscale fibers [18, 19].

To overcome this problem, in our recent study on the PLA–HA, a surfactant was introduced to disperse the HA inorganic particles within the PLA and thus to aid the production into a composite fibrous form [19]. As for the natural polymer–inorganic composite nanofibers, such as the gelatin–HA nanofiber, a recent study was also carried out, wherein, the HA nanocrystallines were firstly mineralized within gelatin, and the produced HA–gelatin nanocomposite sol was used as a precursor for the electrospinning [18]. This approach was proved to aid the dispersion of the HA nanocrystallines within the gelatin matrix and finally to facilitate to obtain a composite nanofibrous structure.

Based on the previous work, we produced herein the collagen–HA nanocomposite nanofiber by the electrospinning method. The processing tools to generate the collagen–HA nanofiber were described and the properties of the nanofiber were examined.

## 2 Materials and methods

### 2.1 Collagen–HA precipitated nanocomposites

Prior to the electrospinning, collagen–HA nanocomposites were prepared by the precipitation process. The precipitation of collagen–HA was based on our previous work [6]. In brief, type I collagen (ateolocollagen from bovine skin, MW 100,000) was dissolved in 59.7 mM phosphoric acid diluted in distilled water. Separately, calcium hydroxide was completely dissolved in distilled water. The P–collagen and Ca solutions were added dropwise into a reaction vessel at 37°C with pH 9. During the co-precipitation reaction, pH

was maintained at 9 using HCl and NH<sub>4</sub>OH. In particular, the concentrations of Ca, P and collagen were determined at the compositions of HA/(collagen + HA) = 20, 30 and 40 wt%. The co-precipitation reaction was lasted for 48 h, and followed by washing with distilled water and lyophilization at –60°C under vacuum.

### 2.2 Electrospinning

The dried collagen–HA nanocomposites were dissolved in an organic solvent 1,1,1,3,3,3-hexafluoro-2-propanol (HFP, from Aldrich) for the preparation of electrospinning sols. The electrospun collagen using HFP was reported to reveal periodic banded appearance which is typical of native collagen [20, 21]. As a pilot study, the optimal sol concentrations for the electrospinning at each composition were examined. For the pure collagen, the optimal concentration was determined at 0.060 g/ml. In the 20%HA added collagen, the spinnability of the sol was examined over the concentration range of 0.023–0.040 g/ml (as summarized in Table 1). Moreover, for the 30% and 40%HA added collagen, the sol concentration was determined at 0.037 g/ml. The nanocomposite sols stirred for 3 days were loaded into a glass syringe (with a capacity of 5 ml and a needle diameter of 500 μm) and injected onto a metal collector under a high DC electric field strength (12 kV/8 cm) at an injection rate of 0.06 ml/h. The electrospun samples were dried under vacuum for 2 days at room temperature to evaporate the remaining solvent.

### 2.3 Characterization

The morphology of the electrospun nanofibers was examined with scanning electron microscopy (SEM, JEOL, Japan). The diameter of the nanofibers was measured from 30 different, arbitrarily selected samples and then averaged. The internal structure of the nanofibers was observed with transmission electron microscopy (TEM, CM20,

**Table 1** Compositions and concentrations of the collagen–hydroxyapatite nanocomposite sols used for the electrospinning

Composition	Sol concentration (g/ml)
Collagen	0.060
20%HA	0.023
	0.027
	0.030
	0.033
	0.037
	0.040
30%HA	0.037
40%HA	0.037

Philips). The chemical analysis of the electrospun nanofibers was conducted with Fourier transform infrared (FT-IR) spectrometer (Nicolet Magma 550 series II, Midac, USA) over the wavelength range of 4000–400  $\text{cm}^{-1}$  at a resolution of 1  $\text{cm}^{-1}$  with an average of 64 scans per sample.

#### 2.4 In vitro cellular assay

For the cell response tests, the electrospun samples were collected onto cover glasses (diameter of 12 mm), which were mounted on a metal collector. The final thickness of the nanofibers was measured to be  $\sim 10$ – $20 \mu\text{m}$ . For the comparison purpose, the dense collagen film was produced by spin-coating the collagen sol on the cover glass. All the specimens were cross-linked using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and *N*-hydroxysuccinimide (NHS) for 3 h, followed by washing fully with distilled water and drying.

Pre-osteoblast cell line MC3T3-E1 was used to observe the cellular behaviors on the nanocomposite nanofibers. The cells were plated onto the specimens at a density of  $1.5 \times 10^4$  cells/ $\text{cm}^2$  and cultured in an osteogenic medium containing 10 mM  $\beta$ -glycerol phosphate and 50  $\mu\text{g}/\text{ml}$  L-ascorbic acid. After harvesting the cells at 2 days, the cell viability was measured as the mitochondrial NADH/NADPH-dependant dehydrogenase activity, using the cell proliferation assay kit (CellTiter 96 Aqueous One Solution, Promega). The culturing medium was removed and 100  $\mu\text{l}$  of assay solution in 1 ml of culture media was added to each well and allowed to react for 3 h. Finally, the colorimetric measurement with 200  $\mu\text{l}$  sample of each solution was performed using a spectrophotometer at 490 nm absorbance.

The cell morphology at day 1 was observed with Confocal Laser Scanning Microscopy (CLSM, LSM 510 NLO, Zeiss) and SEM. For the observation by CLSM, the cells on the samples were washed twice with PBS, fixed in 3.7% formaldehyde solution in PBS for 10 min at room temperature and dyed with Alexa Fluor<sup>®</sup> 546 phalloidin (Molecular Probes, Eugene, Oregon) and ProLong<sup>®</sup> Gold antifade reagent with DAPI (Molecular Probes, Eugene, Oregon). For SEM, the samples were prepared by fixing with glutaraldehyde (2.5%), dehydrating with a graded series of ethanols (75, 90, 95 and 100%), treatment with hexamethyldisilazane (HMDS) and gold coating.

For the assessment of the alkaline phosphatase (ALP) activity, the cells were cultured for 7 and 14 days. After harvesting the cells, the cell pellets were resuspended by vortexing them in 150  $\mu\text{l}$  of 0.1% Triton X-100 and further disrupted by means of cyclic freezing/thawing processes. The cell lysates were quantified using a protein assay kit (BioRad, Hercules, CA) and assayed colorimetrically

for the ALP activity using *p*-nitrophenyl phosphate as a substrate (ALP yellow liquid substrate for ELISA, Sigma, St. Louis, MO) at 405 nm using a microplate reader. The statistical analysis was performed using the Student's *t*-test and significance was considered at  $P < 0.05$ .

### 3 Results

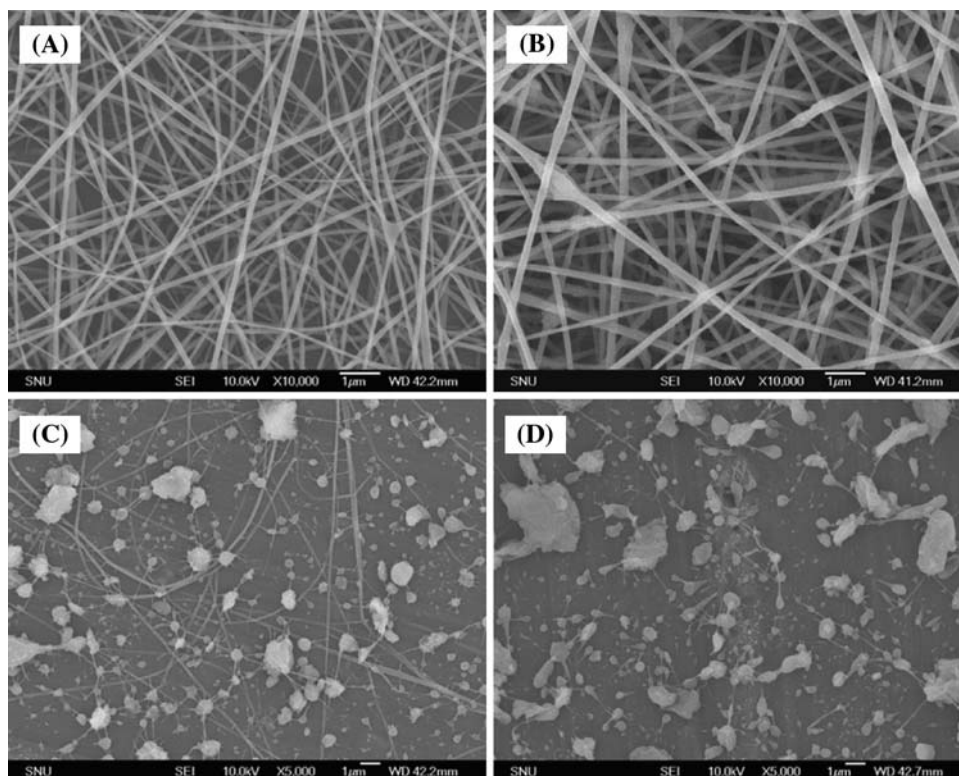
Figure 1 shows the SEM morphologies of the as-electrospun nanofibers of pure collagen and its composites with different amounts of HA (20, 30, and 40 wt%). In particular, the concentrations of sols were fixed at the optimal conditions for each composition which are appropriate for the electrospinning. In the case of pure collagen (Fig. 1a), well-developed nanofibers were generated at a collagen concentration of 0.06 g/ml. No beads were observed showing good spinnability. When HA was added at 20% (Fig. 1b), nanofibers could also be electrospun at a sol concentration of 0.037 g/ml. Although there was no significant difference in the composite fibrous morphology compared to the case of pure collagen, some parts appeared to show the formation of small-sized beads. When the amount of HA was increased to 30% and the sol concentration was maintained at 0.037 g/ml (Fig. 1c), the nanofiber morphology could not be obtained through the electrospinning, showing a significant formation of beads. A similar trend was observed in the case of 40%HA (Fig. 1d).

Using the composition of collagen–20%HA, the effect of sol concentration (range of 0.023–0.040 g/ml) on the spinnability and morphology change of the nanofiber was examined, as shown in Fig. 2. At all concentrations of the nanocomposite sol, well-developed fibrous morphology could be obtained. As the sol concentration increased, the fiber became thicker, and the bead-like formation was less pronounced (Fig. 2a–f). The diameter of the fibers was measured as shown below the SEM images (Fig. 2g). The diameter of the fibers was in the range of  $\sim 70$ – $170$  nm on average.

The internal structure of the collagen–20%HA nanocomposite nanofiber was evaluated with TEM, as presented in Fig. 3. Compared to the homogeneous internal structure observed in the pure collagen nanofiber (Fig. 3a), the nanocomposite was shown to have discrete dark areas within the nanofibers (Fig. 3b). Based on a high magnification image (Fig. 3c), the area was deemed to be the precipitated inorganic phase with highly elongated morphology, moreover, from an electron diffraction pattern the phase was proven to be a poorly crystallized apatite (Fig. 3d).

The FT-IR spectra of the nanofibers were presented in Fig. 4. Along with the amide bands as observed in the pure

**Fig. 1** SEM morphologies of the electrospun nanofibers with varying the amount of HA in collagen: (a) pure collagen, (b) 20%HA, (c) 30%HA and (d) 40%HA. Sol concentrations were fixed at optimal conditions (0.06 g/ml for pure collagen and 0.037 g/ml for nanocomposites). With HA at 20%, nanofibrous webs were generated well, however, above the amount many beads were created



collagen, phosphate bands associated with apatite were well developed in the collagen–20%HA nanofiber.

The cellular responses to the nanocomposite nanofibers were preliminarily assessed using mouse-derived osteoblastic cells. Figure 5 shows the CLSM image of cells attached and grown on the nanofibrous substrates of pure collagen (Fig. 5a, b) and collagen–20%HA (Fig. 5c, d) for 1 day of culturing. On both compositions of nanofiber substrates, the cells were shown to be viable and well-spread with numbers of cytoskeletal extensions.

The cell growth level on the nanofibrous substrates was assessed after culturing for 2 days (Fig. 6a). For the purpose of comparison, the prepared dense collagen film was also tested. The cell growth level was significantly higher ( $P < 0.05$ ) on the nanofibrous collagen than on the dense counterpart with the same composition. Moreover, the nanocomposite nanofiber (collagen–20%HA) showed significantly higher growth level than the dense collagen ( $P < 0.05$ ), but did not show any statistically significant differences with respect to the nanofibrous collagen.

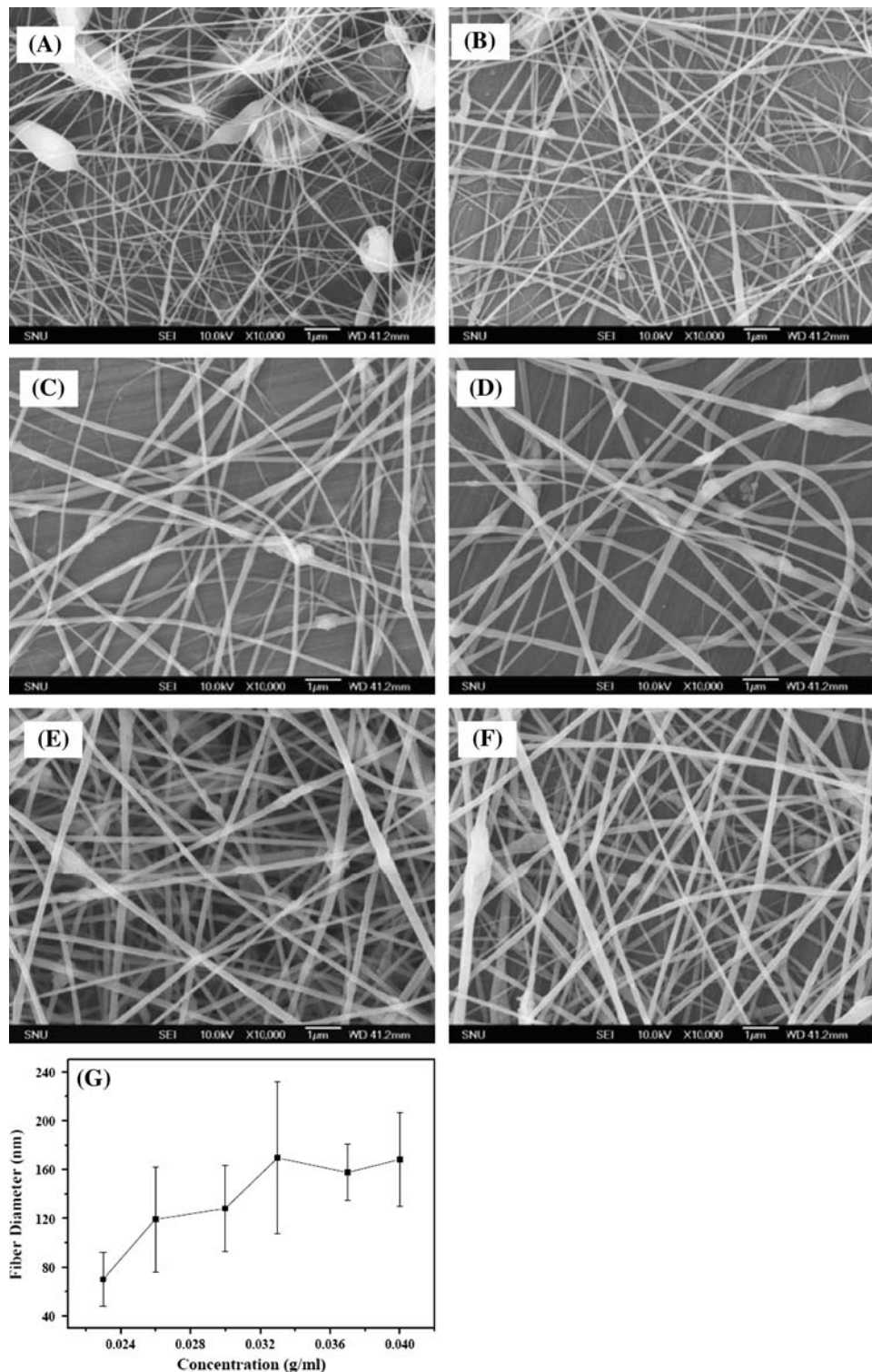
The alkaline phosphatase (ALP) activity expressed by the cells on the two different nanofibrous substrates (pure collagen and collagen–20%HA) was compared, as shown in Fig. 6b. For both compositions, the ALP expression was up-regulated with culturing time. Interestingly, the ALP level at day 7 was significantly higher on the pure collagen, but the level was exceeded by the nanocomposite at day 14.

#### 4 Discussion

The collagen–HA composite has been a promising material choice for the defect augmentation and tissue engineering of bones. In particular, when organized well on the nanoscale, mimicking the composition and structure of bone, the mechanical and biological properties of the composites are known to exceed those of the pure collagen counterpart [6]. Many studies undertaken thus far mainly have concerned with the preparation of nanocomposite in the forms of gel, powder and its pressed bulk, as well as their evaluation of structural, chemical and biological properties. The authors herein developed the collagen–HA nanocomposite system into a nanofibrous web by introducing the electrospinning method. The morphological feature of the nanofiber makes it highly attractive for use as a substrate to populate and support tissue cells. Since the extracellular matrices of connective tissues mainly exhibit a fibrous network of collagenous proteins with sizes of tens to hundreds of nanometers, the electrospun nanofibrous substrates are considered at least to mimic the morphological traits of the tissues.

For the successful generation of the composite into a nanofiber, HA crystallines were first precipitated within the collagen matrix, and the nanocomposite solution was further dried and dissolved in an organic solvent and then subjected to an electrospinning. Through the co-precipitation processing, the HA inorganic component could be produced into ultrafine crystallines and distributed well

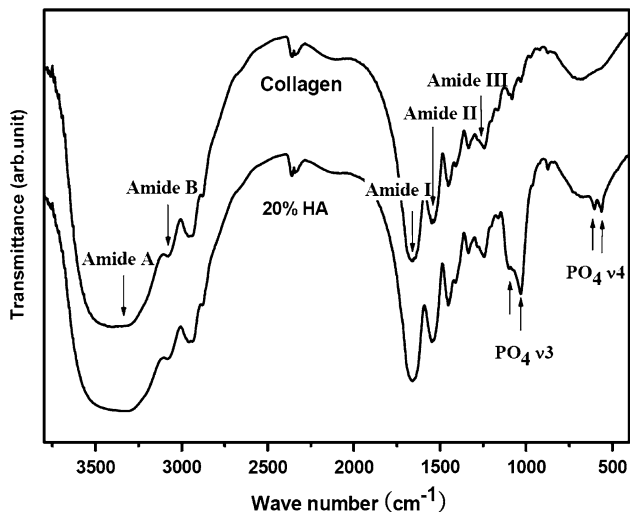
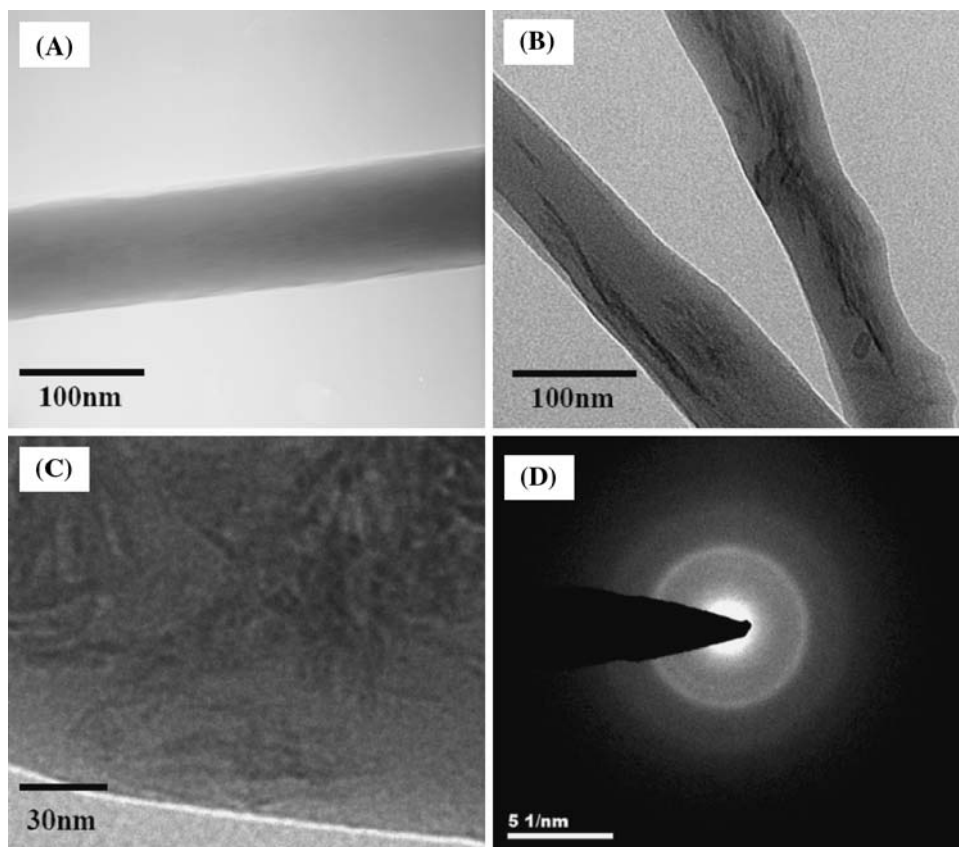
**Fig. 2** (a–f) SEM morphologies of the electrospun collagen–20%HA nanofibers with varying the sol concentration: (a) 0.023 g/ml, (b) 0.027 g/ml, (c) 0.030 g/ml, (d) 0.033 g/ml, (e) 0.037 g/ml and (f) 0.040 g/ml, and (g) diameters of the fibers measured on arbitrary 30 different samples: fiber size increased as the sol concentration was increased. The optimal sol concentration for electrospinning the collagen–20%HA was observed at above 0.037 g/ml



within the collagen organic matrix. It has been recognized that the dispersion of an inorganic component within an organic matrix is of particular importance in the electrospinning of organic–inorganic composites. Other studies concerned with this issue either used diluted inorganic

phase (in the carbon nanotube/polymer system) or introduced surfactant to modify the surface of inorganic phase (in the HA/PLA system) [19, 22]. In our approach of using the co-precipitated sol, the HA inorganic component could be spatially distributed within the collagen organic phase

**Fig. 3** TEM image of the (a) pure collagen and (b) collagen–20%HA electrospun nanofibers, (c) Enlarged image shows the internal structure of the collagen–20%HA nanofiber revealing needle-like apatite nanocrystallines precipitated within the collagen matrix, (d) selected area electron diffraction pattern of the precipitates, showing diffused rings which are characteristics of apatite crystals



**Fig. 4** FT-IR spectra of the pure collagen and collagen–20%HA nanocomposite nanofibers

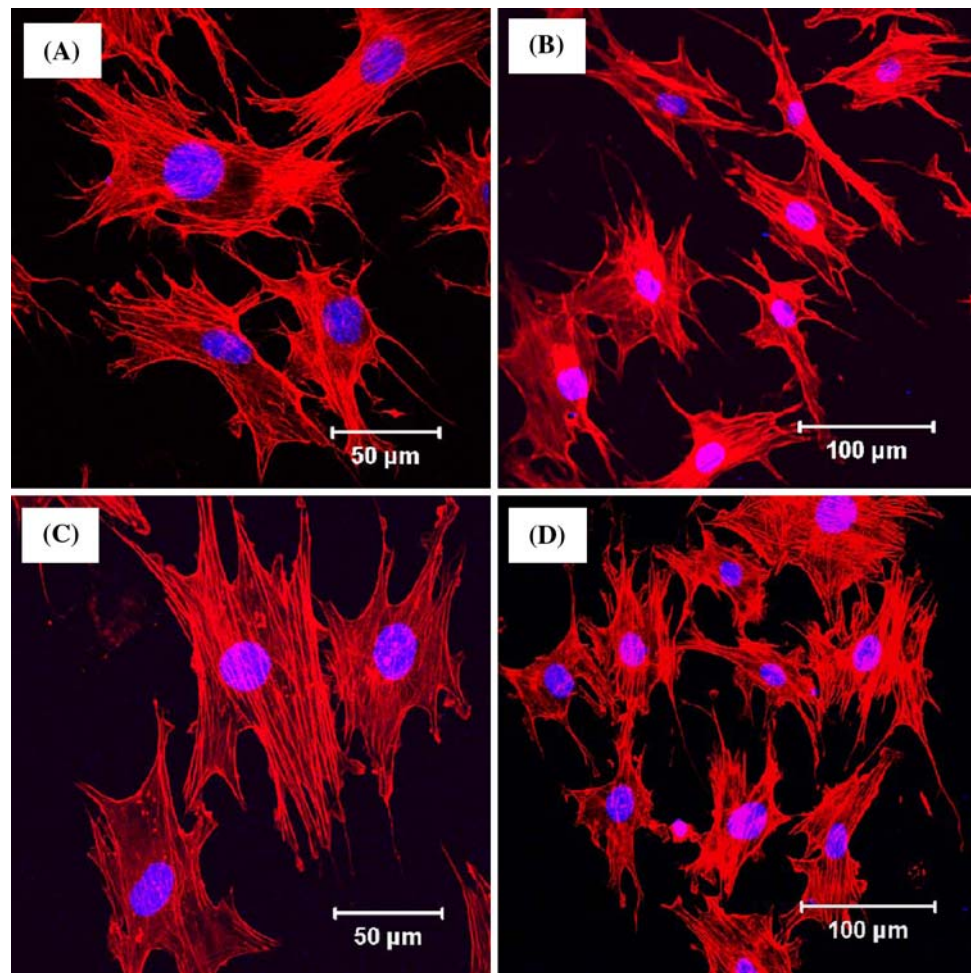
because the collagen amino acid chains could act as a nucleation site for the HA precipitation.

Using the nanocomposite sol, the fibrous form could be obtained with the HA amounts of up to 20 wt% (Fig. 1). However, at the higher HA amounts a considerable level of beads was formed instead of the evolution of a fibrous morphology. It is reasoned that when the HA amount was

high the collagen could not effectively distribute the HA nanocrystallines during the precipitation process due to the relatively low density of the amino acid chains in collagen. As a result of this, some of the HA crystallines could be precipitated in large clusters without the direct involvement of the amino acids of collagen. Although this study shows the HA amount was limitedly loaded (~20 wt%) within collagen, it is further anticipated that a higher level of loading would be possible when such a precipitation process and the electrospinning conditions were improved, and this remains further study.

The nanofibrous substrate showed a clear influence on the cell growth level based on a comparison test with the dense counterpart (Fig. 6a). However, the growth level was not significantly altered depending on the composition (collagen vs. nanocomposite). It has been recognized that the nanofibrous surface improves the adhesion and growth of cells with respect to the dense substrate, by means of increasing the integrin-receptor binding sites for the cellular adhesion and regulating cytoskeletal extensions on the nanoscale [23]. Ma et al. [24] showed the nanofibrous surface made of PLA had better cell adhesion and osteoblastic differentiation than did the PLA dense surface. Matsuda et al. [25] also reported the enhanced endothelial cell adhesion on the polyester substrates with nanofibrous structure than those with the microfibrillar equivalent.

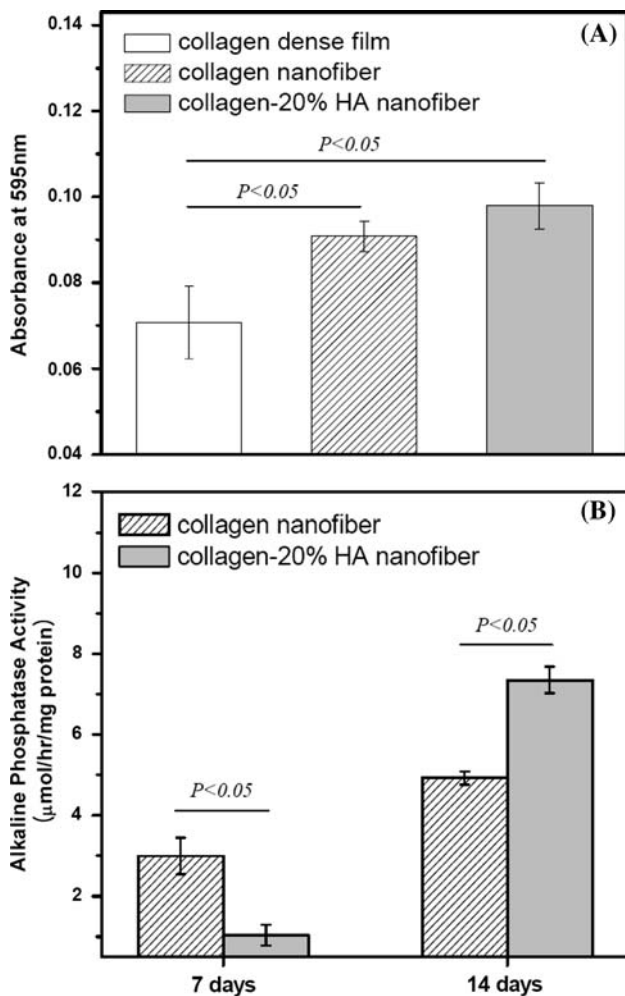
**Fig. 5** Cell growth morphology on the nanofibers: (a, b) pure collagen and (c, d) collagen–20%HA. MC3T3-E1 cells were cultured for 1 day and the nucleus and actin filament of the cells were stained with DAPI and Alexa-546



Although some other physico-chemical parameters associated with the electrospinning process should be considered, the enhanced cell adhesion on the nanofibrous collagen substrate was deemed to be significantly affected by the morphological trait. However, between the two compositions of nanofibrous substrates (collagen and collagen–20%HA) no statistical significance was observed. Based on the study herein, it is considered that the morphological trait had higher impact on the cell growth than did the variation in composition.

Along with the cell growth, the ALP level expressed by the cells was measured to assess the degree of osteoblastic differentiation on the substrate. It was observed that the ALP level was up-regulated with culturing time on both substrates of the nanofibers, and this has been generally observed in the cells which underwent osteoblastic differentiation. Interestingly however, the ALP level was stimulated differently on both substrates in a time-dependent manner. As a result, while only a slight increase was observed on the collagen nanofiber with time from 7 to 14 days, there was considerable increase in the nanocomposite nanofiber with the same time change. It was possible

that when supported on the nanocomposite nanofibrous substrate the murine-derived preosteoblast MC3T3-E1 could be triggered to elicit osteoblastic trait at relatively later time but to a greater extent than on the collagen equivalent. However, owing to the limitation of the current study, any general conclusions cannot be drawn regarding this, particularly as to the impact of the nanocomposite composition on the expression of ALP by the osteoblastic cells with respect to the pure collagen. Although showed some down-regulation in ALP at day 7, the HA–collagen nanofiber stimulated cells to express ALP at a significant incremental rate at day 14, and this may be beneficial for the later mineralization and maturation [26, 27]. At least however, the currently developed nanofibrous web constituted of collagen and HA was proven to support good cell adhesion and growth and to stimulate osteoblastic differentiation, and thus is considered to have some potential to be useful in the bone regeneration field. Further works warrant as to the in-depth investigations of osteoblastic differentiation on the nanocomposite nanofiber as well as its response within animal models for medical applications.



**Fig. 6** Comparison of cellular responses to the nanofibrous substrate: (a) cell proliferation at day 2 assessed by MTS method. Data on dense collagen film are compared with the electrospun nanofibrous substrates. (b) Alkaline phosphatase activity expressed by the cells on both the nanofibrous substrates (collagen and collagen–20%HA) at day 7 and 14. One-way ANOVA followed by Bonferroni analysis showed statistical significance between test groups ( $P < 0.05$ )

## 5 Conclusions

Nanofibrous webs constituted of collagen–HA nanocomposite were produced through the electrospinning technique. A well-developed fibrous morphology could be generated with the HA amounts of up to 20 wt% within collagen. The nanocomposite nanofiber was observed to provide matrix conditions for cells to adhere and populate, in a comparable level to the pure collagen nanofiber, and to direct cells to

elicit osteoblastic marker, alkaline phosphatase. This study suggests the collagen/HA nanocomposite nanofibrous web may be useful as a novel bone regeneration matrix.

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