Magnetic Resonance Functional Nano-Hydroxyapatite Incorporated Poly(Caprolactone) Composite Scaffolds for *In Situ* Monitoring of Bone Tissue Regeneration by MRI

Nitya Ganesh, PhD,¹ Anusha Ashokan, MSc,¹ Ramiah Rajeshkannan, MBBS, MD,² Krishnaprasad Chennazhi, PhD,¹ Manzoor Koyakutty, PhD,¹ and Shantikumar V. Nair, PhD¹

In this study, we have reported the incorporation of a multi-modal contrast agent based on hydroxyapatite nanocrystals, within a poly(caprolactone)(PCL) nanofibrous scaffold by electrospinning. The multifunctional hydroxyapatite nanoparticles (MF-nHAp) showed simultaneous contrast enhancement for three major molecular imaging techniques. In this article, the magnetic resonance (MR) contrast enhancement ability of the MF-nHAp was exploited for the purpose of potentially monitoring as well as for influencing tissue regeneration. These MF-nHAp containing PCL scaffolds were engineered in order to enhance the osteogenic potential as well as its MR functionality for their application in bone tissue engineering. The nano-composite scaffolds along with pristine PCL were evaluated physico-chemically and biologically *in vitro*, in the presence of human mesenchymal stem cells (hMSCs). The incorporation of 30–40 nm sized MF-nHAp within the nanofibers showed a substantial increase in scaffold strength, protein adsorption, proliferation, and osteogenic differentiation of hMSCs along with enhanced MR functionality. This preliminary study was performed to eventually exploit the MR contrast imaging capability of MF-nHAp in nanofibrous scaffolds for real-time imaging of the changes in the tissue engineered construct.

Introduction

HYDROXYAPATITE (HAp) has been one of the most promising scaffold materials for bone tissue engineering, because it is a constituent of natural bone, is osseous inductive,^{1,2} and imparts good mechanical strength. Hence, investigators have attempted to use porous HAp blocks as tissue engineering scaffold constructs^{1,3}; however, such constructs are brittle^[ref] and further do not show any X-ray or MRI contrast and, therefore, the bone defect cannot be monitored in real time during the healing process. Composites of HAp with tougher materials^{4–6} have been investigated for their superior toughness, but these materials also cannot be monitored clinically. Recently, in our labs, we have developed contrast capable multifunctional nano hydroxyapatite (MF-nHAp) particles, which not only have the excellent properties of natural HAp but also have the capability of contrast in several modes, such as MRI, X-Ray, and fluorescence.⁷ In this study, we have explored the application of this novel ceramic filler as a constituent material of scaffolds for bone tissue engineering. The question we have asked is whether this contrast capable HAp can provide the mechanical

and biological functionality while simultaneously being able to provide the contrast capability to the scaffold that will allow the regenerative healing process to be monitored. Toward this end, we have utilized a PCL/MF-nHAp composite scaffold by electrospinning. Electrospun nanofibers help in mimicking the ECM of native bone, which is made up of collagen fibers.^{8,9} We have previously reported¹⁰ the application of electrospun nanofibrous scaffold containing various ceramic fillers^{11,12} for bone tissue engineering. This study will focus on the MR-functional nHAp as a candidate filler in PCL and investigate its ability to provide a superior scaffold for bone tissue regeneration and also to monitor the regeneration of bone.

Materials

The MF-nHAp used in our work was doped with gadolinium to enhance MR contrast.¹³ Details of this material can be found from our previous study.⁷ The size of the nHAp and MF-nHAp is approximately 30–40 nm and the material has contrast capability in three modes: X-Ray, MRI, and fluorescence. Only the MRI contrast is explored in this article. PCL (Polysciences, Inc.) with a number average molecular weight of

¹Amrita Centre for Nanosciences and Molecular Medicine, Amrita Institute of Medical Sciences and Research Centre, Amrita Vishwa Vidyapeetham University, Kochi, India.

²Department of Radiology, Amrita Institute of Medical Sciences, Kochi, India.

43,000–50,000 was used for this study. Chloroform (Merck) and methanol (Merck) were used as solvents. For human mesenchymal stem cells (hMSC) culture, Iscove's modified Dulbecco's medium (IMDM), MSC-specific fetal bovine serum (MSC-FBS), penicillin-streptomycin, and Trypsin-EDTA were purchased from Invitrogen. Cell viability and proliferation assays were done with alamarBlue® (Invitrogen). Proliferation assays also involved MTS (Promega) and PicoGreen™ (Invitrogen). For fixing cells before analyzing samples under a microscope, glutaraldehyde (Fluka) or paraformaldehyde (Sigma) were used. Serum protein adsorption assay was done using bicinconinic acid (BCA), copper sulfate, and bovine serum albumin (BSA) (Sigma) and for testing the ALP activity, ALP enzyme (HiMedia) and p-nitro phenol phosphate liquid substrate (Sigma) were used. Differentiation medium used for differentiation studies was IMDM supplemented with dexamethasone, ascorbic acid, and beta-glycerol phosphate. Fluorescent stains, phalloidin conjugated to Texas Red (Sigma), propidium iodide (PI), and 4, 6-Diamidino-2-phenylindole (DAPI) (Sigma) were used for confocal imaging. Primary antibodies of Ki-67 and *RUNX2* and FITC-conjugated anti-mouse secondary antibody were purchased from (Abcam).

Methods

Fabrication of nano-composite scaffolds

The mixture of chloroform and methanol has been used as a solvent for electrospinning of PCL.³ PCL (12%w/v) was dissolved in 3:2 (v/v) of chloroform: methanol with constant stirring at room temperature. For electrospinning, nHAp and MF-nHAp were mixed with PCL solution and stirred for 1 h to obtain homogeneous solution and sonicated for 5 min in a bath sonicator (Misonix). For preparing nanofibrous scaffolds, the solution was taken in a 10 mL plastic syringe (BD Discardit II) with a blunt end needle tip and a voltage of 16 kV (Gamma High Voltage Research) was applied between the needle tip and the ground collector with a tip-target distance of 12 cm. The flow rate of the solution was optimized at 0.7 mL/h for obtaining nanofibers. The target was an aluminum sheet on which the scaffold was electrospun. PCL, PCL/nHAp, and PCL/MF-nHAp were the different nanofibrous scaffolds fabricated. The percentage of nHAp and MF-nHAp incorporated in the scaffolds was 2.67% (w/w) of PCL. The actual loading of nHAp and MF-nHAp in the composite was found to be ~2.43% using gravimetric analysis.

Characterization

Scanning electron microscope. The structural morphology of fabricated scaffolds was examined using scanning electron microscope (SEM). Nanocomposite scaffolds fabricated by electrospinning were placed on an aluminum stub and coated with gold using the sputter coating unit (JEOL JFC 1600) for 2 min at 10 mA before imaging. The average fiber diameter was determined by measuring the diameter of fibers in a few areas from five SEM images of each scaffold.

Contact angle measurement. Contact angle of the fabricated scaffolds was examined using Drop shape analyzer (KRÜSS-DSA100). All the samples, PCL, PCL/nHAp, and PCL/MF-nHAp, were tested in triplicate. Temporal images

of the solution droplet were automatically taken. From these images, the contact angle values of scaffolds with milli-Q water were calculated using the computer simulation software. The chosen experimental conditions were 25°C and about 65% humidity.

Mechanical testing. Samples were prepared in dimensions of 6 cm length and 2 cm breadth. Scaffolds were uniaxially tested to failure at an extension rate of 10 mm min⁻¹ with an ORIENTEC Universal testing Machine STA-1150 RTC. Four specimens were considered for each electrospun scaffold. The recorded data were used to assess tensile strength with regard to each fiber system.

Leaching studies of gadolinium using inductively coupled plasma-atomic emission spectroscopy

Equal weights (7.5 mg) of PCL, PCL/nHAp, and PCL/MF-nHAp were weighted and incubated in IMDM medium for 1–4 days. The supernatant medium from 1, 2, 4, and 21 day time points was analyzed for the concentration of elemental gadolinium using inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Thermo Electron IRIS INTREPID II XSP DUO; Thermo Scientific).

Serum protein adsorption studies

Adsorption of serum proteins to nano-composite fibers and PCL fibers on scaffold were evaluated over time. Equal weights (7.5 mg) of scaffolds were taken and further cut into small pieces for studies. Scaffolds were placed in a 24-well plate, to which medium (IMDM with 20% MSC-FBS) was added and incubated for varying periods (2, 4, 6, and 8 h) as required. After the incubation of scaffolds in media, they were washed twice with phosphate-buffered saline (PBS). Four-hundred micro liter of elution buffer (0.025% sodium dodecyl sulfate) (Sigma) was added to every scaffold in order to elute the protein adsorbed from serum in medium. After 2 h of incubation in elution buffer, sample and BCA working reagent were added in a ratio of 1:8, mixed well, and incubated for 30 min at 37°C. A BSA solution of 1 mg/mL was made and serially diluted with elution buffer in order to get a standard curve to obtain the unknown protein values adsorbed on scaffolds. The amount of protein in standards as well as samples were found using BCA assay. Plate was read using a microplate spectrophotometer at 562 nm.

Mineralization studies on composite scaffolds

These PCL/nHAp and PCL/MF-nHAp composite scaffolds of 1 cm × 1 cm × 0.2 mm were immersed in SBF solution for a period of 21 days at 37°C. The SBF was prepared using the method developed by Kokubo *et al.*¹⁴ After soaking for a predetermined time, these scaffolds were rinsed with milliQ water (Millipore), blotted, and dried at room temperature. The samples were then characterized by SEM, an energy-dispersive spectrometer, and fourier transform infrared spectroscopy (Perkin-Elmer RX1).

Cell viability of hMSCs on scaffolds

The cell viability assay was done using alamar Blue reagent. Briefly, UCB-hMSCs (Passage 3) were seeded onto nano-composite scaffolds with 8 × 10³ cells per scaffold. All samples were sterilized by exposing ethylene oxide gas at

40°C for 3 h. After 48 h incubation, the medium was replaced by 10% alamar Blue in Dulbecco's modified Eagle medium (Phenol red-). After 8 h of incubation, the plate was read using a microplate spectrophotometer (Biotek Power-Wave XS) at 570 nm with 600 nm set as the reference wavelength. The positive control was PCL nanofibrous scaffold.

Cell proliferation

Proliferation of the hMSCs on PCL, PCL/nHAp, and PCL/MF-nHAp was done by alamarBlue assay for approximately 6 days using the same protocol as mentioned earlier. The proliferation on PCL/MF-nHAp scaffolds was further confirmed by MTS assay, DNA quantification by picogreen, and Ki-67 staining. MTS assay was performed using the same protocol as alamarBlue. MTS reagent was added after the incubation time and incubated for 2 h. The plate was read at 490 nm. For DNA quantification, 10^4 cells were seeded per scaffold and protocol was followed as per the kit specifications. Ki-67 is a protein expressed in all the dividing cells. Hence, it was stained to confirm proliferation on the scaffolds. hMSCs after seeding on the scaffolds were allowed to attach and were serum deprived overnight (≈ 14 h), after which the samples were fixed and permeabilized in 100% methanol for 10 min at -20°C . Samples were blocked using 10% anti-goat serum for 1 h, treated with the Ki-67 primary antibody overnight at 4°C , and washed thrice with PBS. Cells were treated with secondary antibody for 1 h and again washed thrice in PBS. Cells were mounted with a mountant containing nuclear stain (propidium iodide).

Cell attachment studies

hMSCs (5×10^3 /scaffold) were seeded on composite scaffolds that were placed in sterile 24-well tissue culture polystyrene dishes in stationary conditions. These scaffolds were sterilized using ethylene oxide gas at 40°C for 3 h, before seeding cells. Scaffolds with seeded cells were conditioned with IMDM containing 20% MSC-FBS for 12 h. After incubation, samples were fixed and analyzed by SEM. Fixing was done with 0.25% glutaraldehyde for an hour at 4°C . The specimens were thoroughly washed and dehydrated using ethanol gradient, gold sputtered in vacuum, and examined using SEM.

Differentiation of hMSCs on PCL/HAp scaffolds

ALP activity was measured based on the hydrolysis of p-nitrophenyl phosphate liquid substrate to p-nitrophenol. All

scaffolds after seeding with hMSCs (10^4 /scaffold), were cultured in both normal medium and osteogenic medium as required. Fifty percent of medium was replenished every other day. Scaffolds with cells were incubated for time durations of 1, 7, 14, 21 days as required. The cell lysates were prepared by treating with 1% Triton X-100 for 1 h. ALP substrate and glycine buffer were taken in a microtiter plate to which cell lysate (enzyme) was added with the lysate: substrate ratio being 1:1. The plate was incubated in the dark for 30 min at 37°C . After incubation, 5M NaOH was added to stop the enzymatic reaction, read using a microplate spectrophotometer, and measured at 405 nm. The ALP activity was determined using the standard graph obtained by serial dilutions of ALP enzyme. Later, the values were normalized with the total protein amount (per reaction) measured by the BCA assay using the same protocol as mentioned earlier.

Expression of *RUNX2* protein was checked by immunocytochemistry. The scaffolds were seeded with hMSCs and incubated for 1–21 days as done earlier for ALP studies. These samples at various time points were fixed and permeabilized in 100% methanol for 10 min at -20°C . Later, they were blocked using 1%FBS for 30 min, followed by staining with *RUNX2*-specific primary antibody, and incubated overnight at 4°C . Samples were washed twice in PBS and incubated for 1 h with the secondary antibody. They were again washed twice with PBS and counter-stained with DAPI to stain the nuclei. Samples were analyzed using a confocal microscope (Leica; SP 5 II). Images were taken using $20\times$ magnification and with $2.8\times$ zoom.

MRI imaging by T_1 -weighted contrast of the scaffolds

MR imaging experiments were performed with a 1.5 T clinical scanner (GE Health Care). T_1 contrast images were recorded by pulse sequence spin-echo technique with TR/TE = 200 ms/9 ms, FOV 19 by 19 cm^2 , resolution 256–256 points, four acquisitions, and band width 41.67 KHz. The three electrospun scaffolds PCL, PCL/nHAp, and PCL/MF-nHAp were stuck on 18 mm round coverslips and embedded in 2% agar in a 12-well plate, and the T_1 contrast images were captured without cells. These three scaffolds were seeded with hMSCs (10^4 cells/scaffold) and incubated in osteogenic medium for 7, 14, and 21 days. At these time points, the scaffolds were embedded in 2% agar and analyzed using MRI to investigate the contrast changes at the various time points during the regenerative process.

FIG. 1. Electrospun nanofibrous scaffolds. Scanning electron micrographs showing (A) PCL, (B) PCL/nHAp, and (C) PCL/MF-nHAp electrospun nanofibrous scaffolds. PCL, poly(caprolactone); MF-nHAp, multifunctional nano hydroxyapatite.

Statistical analysis

All quantitative results were obtained from experiments done in triplicate. Data was expressed as the Mean $\pm$ SD. Statistical analysis was carried out using a Student's two-tailed *t*-test. A *p*-value of ≤ 0.05 was considered statistically significant.

Results

Characterization of PCL/nHAp and PCL/MF-nHAp nanocomposite fibrous scaffold

SEM images in Figure 1 show the electrospun nanofibrous scaffolds of PCL, PCL/nHAp, and PCL/MF-nHAp. The obtained nanofibers were randomly oriented and were in the range of 100–500 nm in diameter. The incorporation of the HAp particles slightly decreased the diameter of the fibers (Fig. 1B), while the MF-nHAp incorporation delivered fibers of smaller diameters to that of native PCL (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

Contact angle measurement

The contact angle was measured with milli-Q water in order to check the hydrophilicity/hydrophobicity of the scaffold. PCL on its own showed a contact angle of $146.2 \pm 0.6^\circ$ (Table 1), whereas the fabricated composite scaffolds showed a smaller contact angle, namely, $137.5 \pm 1.2^\circ$ and $130.8 \pm 1.0^\circ$, respectively, for PCL/nHAp and PCL/MF-nHAp (Table 1). The measured contact angle value reflected the decreased hydrophobicity of the composite scaffolds compared with PCL.

Mechanical testing

Tensile stress at failure of electrospun native PCL scaffold was found to be 1.3 MPa, and that of PCL/nHAp was found to be 1.7 MPa (Fig. 2). Stress at failure of PCL/MF-nHAp scaffold was as high as 3.35 MPa. Hence, the composite scaffolds showed a higher mechanical strength compared with native PCL.

Leaching studies of gadolinium from the scaffolds

ICP-AES analysis detected only a very small amount of gadolinium in the medium containing the PCL/MF-nHAp scaffold. The spectroscopy results showed 0.2 ppm of gadolinium leaching out on day 1, 0.23 ppm on day 2, 0.27 ppm on day 4, and 3.8 ppm on day 21 (Table 2). The other two scaffolds, PCL and PCL/nHAp did not show any presence of gadolinium as expected.

TABLE 1. CONTACT ANGLE MEASUREMENTS OF ELECTROSPUN COMPOSITE SCAFFOLDS

Sample	Contact angle (θ)
PCL	146.2 ± 0.6
PCL/nHAp	137.5 ± 1.2
PCL/MF-nHAp	130.8 ± 1.0

Table showing contact angle with milli-Q water of PCL, PCL/nHAp, and PCL/MF-nHAp nanofibrous scaffolds. Values represent mean $\pm$ SD of three independent experiments.

PCL, poly(caprolactone); MF-nHAp, multifunctional nano hydroxyapatite.

FIG. 2. Mechanical testing of the nano-composite scaffolds. Graph showing tensile strength of the three electrospun nano-composite scaffolds, namely PCL, PCL/nHAp, and PCL/MF-nHAp (*shows significance compared with PCL, with $p \leq 0.05$).

Protein adsorption studies

The protein adsorption of the composite scaffolds incubated in culture media at different time intervals was assessed by BCA assay. The incorporation of nHAp and MF-nHAp into the scaffolds significantly improved the amount of protein adsorbed after 8 h on PCL/nHAp and PCL/MF-nHAp scaffolds. Values were $9.5 \pm 0.22 \mu\text{g}/\text{mg}$ scaffold in PCL/nHAp and $11.5 \pm 0.38 \mu\text{g}/\text{mg}$ scaffold in PCL/MF-nHAp scaffolds compared with PCL, which was $8.6 \pm 0.24 \mu\text{g}/\text{mg}$ scaffold (Fig. 3).

In vitro mineralization studies of scaffolds

Mineralization studies showed that on the 21st day after incubating in SBF, there were mineral deposits on both the composite scaffolds (Fig. 4A, C). The Ca/P ratios of the mineral deposits on PCL/nHAp and PCL/MF-nHAp scaffolds were found to be 1.802 and 1.9, respectively (Fig. 4B, D). The Ca/P ratios of the nHAp and MF-nHAp were ≈ 1.65 .

TABLE 2. INDUCTIVELY COUPLED PLASMA-ATOMIC EMISSION SPECTROSCOPY ANALYSIS AFTER INCUBATING SCAFFOLDS IN MEDIUM

Sample	Time interval (No. of Days)	Gd (ppm)	Standard deviation (ppm)
PCL	1	BDL	
PCL/nHAp	1	BDL	
PCL/MF-nHAp	1	0.2	± 0.00
PCL	2	BDL	
PCL/nHAp	2	BDL	
PCL/MF-nHAp	2	0.23	± 0.015
PCL	4	BDL	
PCL/nHAp	4	BDL	
PCL/MF-nHAp	4	0.27	± 0.02
PCL	7	BDL	
PCL/nHAp	7	BDL	
PCL/MF-nHAp	7	0.29	± 0.07
PCL	21	BDL	
PCL/nHAp	21	BDL	
PCL/MF-nHAp	21	3.8	± 0.2

Table showing the concentration of gadolinium leached out from the scaffolds over time (1–21 days).

BDL, below detectable limit (i.e., 0.1 ppm).

FIG. 3. Protein adsorption on nanofibrous scaffolds. Graph shows the total protein adsorbed on PCL/nHAp and PCL/MF-nHAp scaffolds compared with PCL nanofibers within 2–6 h of duration. Values represent mean $\pm$ SD of three independent experiments (*shows significance compared with PCL, with $p \leq 0.05$).

Cell viability by alamarBlue on scaffolds

The cytocompatibility of the composite scaffolds was checked by direct contact method. Alamar Blue assay results at 48 h show that the PCL/nHAp and PCL/MF-nHAp scaffolds have 98% and 120% viability (Fig. 5A) of hMSCs, respectively, compared with PCL scaffolds. Both the composite scaffolds were thus found to be biocompatible. The PCL/MF-nHAp scaffolds showed a significant rise in the viability at 48, 96, and 144 h time points (Fig. 5B). On day 1, scaffolds show 10,000 cells per scaffold and on day 6, the number of cells rises to approximately 82,000 per scaffold on PCL/MF-nHAp compared with 60,000 on PCL/nHAp. The rate of proliferation of hMSCs on the PCL and PCL/nHAp scaffolds was found to be similar (Fig. 5B).

Proliferation studies

The alamar Blue assay results show that the PCL/nHAp scaffolds show a higher rate of proliferation as compared with PCL scaffolds. To confirm whether the PCL/MF-nHAp scaffolds support proliferation, MTS assay and PicoGreen assay were done. The PCL/MF-nHAp scaffolds showed a significantly higher rate of proliferation compared with PCL

FIG. 4. Scanning electron microscopy (SEM) images and EDS graphs of mineralized composite scaffolds. SEM images of (A) PCL/nHAp and (C) PCL/MF-nHAp mineralized scaffolds after 21 days in SBF. EDS graphs of (B) PCL/nHAp and (D) PCL/MF-nHAp mineralized scaffolds after 21 days in SBF showing the Ca/P ratios. EDS, energy-dispersive spectrometer. Color images available online at www.liebertpub.com/tea

FIG. 5. Cell viability studies on scaffolds by alamarBlue. **(A)** Cell viability of hMSCs on scaffolds compared with their respective controls after 48 h and **(B)** Proliferation of hMSCs for a period of 1–6 days. Values represent mean $\pm$ SD of three independent experiments (*shows significance compared with PCL, with $p \leq 0.05$). hMSCs, human mesenchymal stem cells. Color images available online at www.liebertpub.com/tea

scaffolds by both MTS assay and DNA quantification assay, as shown in Figure 6. Apart from that, the protein Ki-67 was also expressed in more number of cells on PCL-MF-nHAp scaffolds compared with PCL scaffolds (Fig. 6C, D). On PCL/MF-nHAp scaffolds, 74.4% cells were positive for Ki-67 and only 25% cells were positive for Ki-67 on PCL scaffolds.

Cell attachment and spreading on scaffolds

Cell adhesion studies showed that the hMSCs were attached and well spread on the scaffolds after 12 h in culture (Fig. 7A–C). In both the PCL/nHAp and PCL/MF-nHAp nano-composite scaffolds, the morphology of the cells was flattened and elongated. These results did not appear to be altered by the presence or absence of nHAp in the fibers. The morphologies of hMSCs on composite nanofibers at 24 h are projected in Figure 7D–F by fluorescence staining of actin fibers in red, nuclei in blue, and nanofibers in green. The extended actin filaments show the well-spread cells on the nanocomposite scaffolds. The staining shows that there is a fine spreading of cells on PCL/MF-nHAp scaffolds.

Differentiation of hMSCs on nanocomposite scaffolds

Differentiation of hMSCs, measured by quantifying the amount of ALP enzyme at various time points, was plotted in Figure 8 for various scaffolds (PCL alone and PCL with MF-nHAp) in both normal and osteogenic medium. ALP activity on all scaffolds was below 1 $\mu\text{g}/\text{mg}$ protein on day

1. On day 7 and 14, the ALP activity rose to 2–4 $\mu\text{g}/\text{mg}$ protein on nanofibrous scaffolds in osteogenic medium. On day 21, the ALP activity was significantly high in hMSCs on the scaffolds in osteogenic medium, while on the scaffolds in normal medium, it remained low. The hMSCs on PCL/MF-nHAp scaffolds showed a significant increase in ALP activity (Fig. 8) compared with PCL scaffolds on day 21. The expression of protein *RUNX2* was detected on day 7 in hMSCs on nHAp and MF-nHAp containing scaffolds, while on native PCL scaffold, the expression was found only on day 14 (Fig. 9). All scaffolds showed RunX2 expression on days 14 and 21 after culturing in osteogenic medium.

MRI by T_1 -weighted contrast of the scaffolds

The T_1 -weighted images of PCL scaffold with and without hMSCs were the control samples. PCL/nHAp scaffolds showed no contrast changes compared with the control over a period of 21 days. The PCL/MF-nHAp scaffold without cells showed a bright contrast compared with the native PCL scaffold (Fig. 10). This bright contrast of the scaffold got even brighter after 7 days of hMSCs cultured on them in osteogenic medium. On 14th day after culture of hMSCs, the contrast was the brightest. After the 21st day in differentiating medium, the hMSCs started differentiating and the contrast of the PCL/MF-nHAp reduced and faded to that of control scaffolds (PCL & PCL-nHAp) (Fig. 10). The mean intensities of the samples in MRI were measured in order to get a quantitative value of the brightness and are as shown in Figure 11. The intensity values also show that the brightness of the PCL-MF-nHAp scaffold

FIG. 8. The alkaline phosphatase activity in hMSCs on composite scaffolds. ALP activity of hMSCs on PCL, PCL/nHAp, and PCL/MF-nHAp scaffolds in normal medium (NM) and osteogenic medium (OM) over time (1–21 days). Values represent mean $\pm$ SD and (*shows significance compared with PCL, with $p \leq 0.05$).

FIG. 9. Immunocytochemistry of RunX2 in hMSCs on scaffolds. Fluorescent images (merged) showing hMSCs on (A–C) Day 1, (D–F) 7, (G–I) 14, and (J–L) 21 in osteogenic medium on scaffolds PCL, PCL/nHAp, and PCL/MF-nHAp scaffolds, respectively, stained for RunX2 (red), DAPI (blue), and nanofibrous scaffolds (green). Color images available online at www.liebertpub.com/tea

FIG. 10. MRI by T1-weighted contrast of the scaffolds. The images showing PCL scaffolds (bottom panel), PCL/nHAp scaffolds (middle panel), and PCL/MF-nHAp scaffolds (top panel) with and without hMSCs.

grows after 7 and 14 days of hMSCs in osteogenic medium. The intensity reduced by day 21. This correlates to the bone regeneration taking place. The schematic representation of the changes in the scaffold during bone regeneration and the corresponding MRI is given in Figure 12. This pictorially represents the changes in an MR functional scaffold during bone regeneration.

Discussion

Mechanical strength of the composite scaffolds

Our previous work reported the PCL/nHAp nanofibrous scaffold to be mechanically stronger compared with nanofibrous native PCL scaffolds.¹⁵ The mechanical strength of the baseline PCL/nHAp used as a control in this study was also found to be stronger than PCL, which is consistent with the previous study. The increase in mechanical strength could be attributed to the nHAp, which is known to increase the stiff-

ness and compressive strength of native bone. What is unique here is the role of MF-nHAp in strength. As Figure 2 showed, the strength increased by about 153% with the addition of 2.65 wt.% MF HAp compared with only a 30% increase when the same amount of conventional nHAp was added. This higher strength may be attributed to the smaller PCL diameter (Fig. 1) in the PCL/MF-nHAp composite scaffold (Supplementary Fig. S1). It is expected that for a given volume of fiber deposited, the fiber spacing would scale linearly with the fiber diameter; hence, the pore sizes would also decrease, which can improve strength. These morphological changes induced by MF-nHAp may be the causative factor in the strength increase. The small amounts of doped Gd (6%) are not expected to change the mechanical properties of nHAp but they could influence the PCL-MF-nHAp interface characteristics by providing additional positively charged sites on the surface for interaction with the polymer, which can, in turn, influence strength. It is well known that PCL interacts with Gd by binding to its positively charged sites, forming hydrolyzable ester bonds.¹³ The sizes of the MF nHAp and nHAp are similar as was reported earlier⁷ by us; hence, this factor could not have played a role in the strength increase.

Contact angle of the scaffolds

The decrease of the contact angle (Table 1) by HAp addition to PCL is consistent with the fact that HAp itself is relatively hydrophilic with a contact angle of approximately 65.2 with water as reported earlier¹⁶ compared with about 146 for PCL alone.¹⁷ A study by Chuenjitkuntaworn *et al.*¹⁸ demonstrated that PLLA/HAp composite fiber mats had a lower contact angle compared with PLLA fiber mat and that the HAp particles on the surface of the fiber mats causes the scaffold to be hydrophilic. Similarly, a study on PLGA/nHAp scaffolds also showed that incorporation of nHAp makes the scaffold hydrophilic.¹⁹

Protein adsorption on the composite scaffolds

As shown in Figure 3, the protein adsorption increased due to nHAp addition in the nanofibrous scaffolds. This is consistent with several other studies.^{15,20,21} Ma *et al.*²² have

FIG. 11. Intensities of the scaffolds in MRI image. Graph showing the mean intensities of magnetic resonance (MR) contrast obtained of different samples at various time points. Color images available online at www.liebertpub.com/tea

FIG. 12. Schematic diagram of the contrast changes by MRI during bone regeneration *in vitro*. The picture shows the different stages of bone regeneration and the corresponding changes in the contrast MRI, when PCL/MF-nHAp scaffold is being monitored. Color images available online at www.liebertpub.com/tea

suggested that the irregular surfaces of the nHAp can contribute to enhanced protein adsorption.

Webster *et al.*^{23,24} have shown a significant increase in protein adsorption on a nanosized ceramic surface compared with a micron-sized ceramic surface due to an increase in surface area. This is also consistent with the current results in which nano-sized HAp particles were added to the PCL.

Mineralization on the composite scaffolds

The mineralization studies conducted on the scaffolds showed that there were mineral deposits on both the PCL/nHAp and the PCL/MF-nHAp scaffolds. The mineralization studies with cells (hMSCs) on the scaffold in osteogenic medium also showed mineral deposits after 21 days (Supplementary Fig. S2) which proves that the hMSCs differentiate in osteogenic medium. The Ca/P ratios of the mineral deposits that formed on both the composite scaffolds were found to be ≈ 1.9 (Fig. 4). This could be due to more deposition of tetracalcium phosphate minerals. In a previous study,²⁵ tetracalcium phosphate was proved to have bioresorption behavior. On the other hand, the Ca/P ratio of the incorporated nHAp particles by themselves was approximately 1.65 with the values for MF-nHAp slightly lower than those for nHAp. Thus, the Ca/P ratio for MF-nHAp is closer to that for native bone, namely, 1.631.²⁶ An earlier study²⁷ showed that the incorporation of nHAp could promote and improve the deposition of bone-like apatite crystals on the porous composite scaffolds. Improvement in the amount of deposition and also uniformity of the apatite particle deposition was shown on nHAp/PLLA composite scaffolds, than on pure PLLA scaffolds.

Proliferation studies of hMSCs

The PCL/MF-nHAp scaffolds showed a significant increase in proliferation (Fig. 6) compared with the corre-

sponding nHAp-containing scaffolds. This could be due to the presence of the gadolinium in the nanofiller. As shown in earlier studies,^{28,29} 2–60 μM gadolinium was found to promote proliferation in cells. The gadolinium could promote the cell cycle progression by enhanced entry to the S-phase by activating ERK and PI3K signaling pathways,²⁸ which are essential regulators of cell cycle progression. Fu *et al.*²⁸ showed that the addition of gadolinium to the cells promoted cell cycle progression, where the particles could have entered the cells or mere physical adsorption might have enhanced the proliferation. In our study, 0.2–0.27 ppm of gadolinium was being leached out from the PCL/MF-nHAp scaffolds (Table 2), which could be acting on the proliferation of cells along similar lines. The exact mechanism behind this is yet to be found. Here, the small concentration of 0.01 nM gadolinium doping present in PCL/MF-nHAp was able to induce proliferation in hMSCs (Fig. 6).

Differentiation of hMSCs on the composite scaffolds

The differentiation studies on scaffolds confirmed that the hMSCs could differentiate to osteoblasts on these scaffolds. The *RUNX2* protein was expressed much earlier on the PCL/nHAp (Fig. 9E) as well as on the PCL/MF-nHAp (Fig. 9F) scaffolds compared with native PCL (Fig. 9D), which is consistent with the results seen in previous studies with nHAp.^{30,31} This proves that the osteogenic potential of hMSCs was higher on the scaffolds containing nHAp^{5,32} and MF-nHAp nanoparticles. ALP activity is found in both preosteoblasts and osteoblasts. The ALP activity measured in this study had a similar trend to that seen in our previous study,¹¹ which showed enhanced osteogenic potential of hMSCs on composite nanofibrous scaffolds. The hMSCs had a significantly higher osteogenic potential on MF-nHAp-containing scaffolds. This is the first study which suggests that hMSCs may differentiate more readily when exposed to MF-nHAp within the PCL nanofibers.

Monitoring the stages of regeneration by MRI

MRI can provide high-resolution information about the functional properties of the newly grown tissue such as the presence on inflammation, edema, and tissue damage due to necrosis/apoptosis compared with X-ray imaging. Due to higher sensitivity, MRI may give better understanding on the degree of tissue integration with the scaffold. The MF-nHAp particles were doped with Gd^{3+} , which is paramagnetic and possess high magnetic moment due to isotropic electronic ground state $^8S_{7/2}$ and half-filled f-orbital. This leads to large effects on both longitudinal and transverse proton relaxivities of Gd^{3+} even at low applied magnetic fields.³³ The scaffold containing this MF-nHAp showed contrast changes by MRI over the regeneration period (Fig. 10). The changes taking place in an implant during *in vitro* bone regeneration is schematically shown in Figure 12. Here, we also show the hypothetical change in MR contrast at various stages. The native PCL scaffold is hydrophobic and hence will enable very less proton exchange with the physiological liquid around the scaffold, resulting in a dark image. In the MF-nHAp incorporated PCL sample without cells, the hydrophobicity of the polymer rendered poor proton exchange between contrast agent and water (Fig. 10). When cells start growing and proliferating on these scaffolds, the contrast increase rendered a bright image by day 7 and 14. This indicates that when the cells are in contact with the scaffold, the hydrophobic polymer interacts better with the lipid content³⁴ of the cell membrane, thereby facilitating a better proton exchange with the nano contrast agent (MF-nHAp). Interestingly the MR contrast of scaffolds peaked at day 14 and decreased (Fig. 11) as minerals started depositing and tissue started developing,^{35,36} by day 21. The clear implication of this is that the contrast is dependent on the stage of regeneration of the tissue and, hence, this provides the possibility of monitoring tissue regeneration. The present composite provides this useful diagnostic capability while simultaneously maintaining the excellent bone forming capacity and strength of the bone.

Conclusions

The MF-nHAp-containing scaffolds were mechanically stronger than PCL/nHAp scaffolds. PCL/MF-nHAp scaffolds were biocompatible and promoted the proliferation of hMSCs compared with PCL and PCL/nHAp scaffolds. The nHAp and MF-nHAp improved the bioactivity of the scaffolds and supported early osteogenic differentiation of hMSCs. Hence, PCL/MF-nHAp was found to be a very good scaffolding material for bone tissue engineering. Apart from this, the MR functionality of the PCL/MF-nHAp scaffold was an added advantage. These scaffolds could be used in monitoring the changes of the engineered construct *in vitro* during bone regeneration, thus enabling researchers in ascertaining the stage of bone regeneration and in determining when the engineered construct would be ready for transplantation.

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Disclosure Statement

No competing financial interests exist.

References

1. Chen, Q.Z., Wong, C.T., Lu, W.W., Cheung, K.M.C., Leong, J.C.Y., and Luk, K.D.K. Strengthening mechanisms of bone bonding to crystalline hydroxyapatite *in vivo*. *Biomaterials* **25**, 4243, 2004.
2. Jardo, M., Kay, J.F., Kumar, K.I., Doremus, R.H., and Drobeck, H.P. Tissue cellular and subcellular events at a bone-ceramic hydroxyapatite interface. *J Bioeng* **1**, 79, 1977.
3. Zhou, H., and Lee, J. Nanoscale hydroxyapatite particles for bone tissue engineering. *Acta Biomater* **7**, 2769, 2011.
4. Chen, B., and Sun, K. Mechanical and dynamic viscoelastic properties of hydroxyapatite reinforced poly(ϵ -caprolactone). *Polym Test* **24**, 978, 2005.
5. Chuenjitkuntaworn, B., Inrung, W., Damrongsri, D., Mekapiruk, K., Supaphol, P., and Pavasant, P. Polycaprolactone/Hydroxyapatite composite scaffolds: Preparation, characterization, and *in vitro* and *in vivo* biological responses of human primary bone cells. *J Biomed Mater Res* **94A**, 241, 2010.
6. Thomas, V., Dean, D.R., Jose, M.V., Mathew, B., Chowdhury, S., and Vohra, Y.K. Nanostructured biocomposite scaffolds based on collagen coelectrospun with nanohydroxyapatite. *Biomacromolecules* **8**, 631, 2007.
7. Ashokan, A., Menon, D., Nair, S.V., and Koyakutty, M. A molecular receptor targeted hydroxyapatite nanocrystal based multi-modal contrast agent. *Biomaterials* **31**, 2606, 2010.
8. Vasita, R., and Katti, D.S. Nanofibers and their applications in tissue engineering. *Int J Nanomed* **1**, 15, 2006.
9. Liao, S., Murugan, R., Chan, C.K., and Ramakrishna, S. Processing nanoengineered scaffolds through electrospinning and mineralization suitable for biomimetic bone tissue engineering. *J Mech Behav Biomed Mater* **1**, 252, 2008.
10. Binulal, N.S., Menon, D., Nagarajan, S., Shalumon, K.T., Stephen, S., Mony, U., Rangasamy, J., and Nair, S. Role of nano-fibrous poly(caprolactone) scaffolds on human mesenchymal stem cell attachment and spreading for *in vitro* bone tissue engineering-response to osteogenic regulators. *Tissue Eng Part A* **16**, 393, 2009.
11. Nitya, G., Jayakumar, R., Manzoor, K., Mony, U., and Nair, S.V. Embedded silica nanoparticles in poly(caprolactone) nanofibrous scaffolds enhanced osteogenic potential for bone tissue engineering. *Tissue Eng A* **18**, 1867, 2012.
12. Nitya, G., Greeshma, T.N., Mony, U., Chennazhi, K.P., and Nair, S.V. *In vitro* evaluation of electrospun PCL/nanoclay composite scaffold for bone tissue engineering. *J Mater Sci Mater Med* **23**, 1749, 2012.
13. Blanquer, S., Guillaume, O., Letouzey, V., Lemaire L., Franconi, F., Paniagua, C., Coudane, J., and Garric, X. New magnetic-resonance-imaging-visible poly(ϵ -caprolactone)-based polyester for biomedical applications. *Acta Biomater* **8**, 1339, 2012.
14. Kokubo, T., and Takadama, H. How useful is SBF in predicting *in vivo* bone bioactivity? *Biomaterials* **27**, 2907, 2006.

15. Shalumon, K.T., Binulal, N.S., Deepthy, M., Jayakumar, R., Manzoor, K., and Nair, S.V. Preparation, characterization and cell attachment studies of electrospun multi-scale poly(caprolactone) fibrous scaffolds for tissue engineering. *J Macromol Sci A* **48**, 21, 2011.
16. Vandiver, J., Patel, N., Bonfield, W., and Ortiz, C. Nanoscale morphology of apatite precipitated onto synthetic hydroxyapatite from simulated body fluid. *Key Eng Mater* **284**, 497, 2005.
17. Wang, Y., Wang, G., Chen, L., Li, H., Yin, T., Wang, B., Lee, J.C.-M., and Yu, Q. Electrospun nanofiber meshes with tailored architectures and patterns as potential tissue-engineering scaffolds. *Biofabrication* **1**, 9, 2009.
18. Chuenjitkuntaworn, B., Supaphol, P., Pavasant, P., and Damrongsri, D. Electrospun poly(L-lactic acid)/hydroxyapatite composite fibrous scaffolds for bone tissue engineering. *Polym Int* **59**, 227, 2010.
19. Mouthuy, P.-A., Ye, H., Triffitt, J., Oommen, G., and Cui, Z. Physico-chemical characterization of functional electrospun scaffolds for bone and cartilage tissue engineering. *Proc Inst Mech Eng H* **224**, 1401, 2010.
20. Woo, K.M., Zhang, R.Y., Deng, H.Y., and Ma, P.X. Protein mediated osteoblast survival and migration on biodegradable polymer/hydroxyapatite scaffolds. *Trans Soc Biomater* **25**, 605, 2002.
21. Woo, K.M., Wei, G., and Ma, P.X. Enhancement of fibronectin and vitronectin-adsorption to polymer/hydroxyapatite scaffolds suppresses the apoptosis of osteoblasts. *J Bone Miner Res* **17**, M49, 2002.
22. Wei, G., and Ma, P.X. Polymer/ceramic composite scaffolds for bone tissue engineering. In: Ma, P.X., and Elisseeff, J., eds. *Scaffolding in Tissue Engineering*. Florida: CRC Press, Taylor & Francis Group, 2006, pp. 241–249.
23. Webster, T.J., Ergun, C., Dpremus, R.H., Siegel, R.W., and Bizios, R. Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *J Biomed Mater Res* **51**, 475, 2000.
24. Webster, T.J., and Siegel, R.W., Bizios. Osteoblast adhesion on nanophase ceramics. *Biomaterials* **20**, 1221, 1999.
25. Tsai, C.H., Lin, R.M., Ju, C.P., Chern, and Lin, J.H. Bioresorption behavior of tetracalcium phosphate-derived calcium phosphate cement implanted in femur of rabbits. *Biomaterials* **29**, 984, 2008.
26. Samavedi, S., Horton, C.O., Guelcher, S.A., Goldstein, A.S., and Whittington, A.R. Fabrication of a model continuously graded co-electrospun mesh for regeneration of the ligament–bone interface. *Acta Biomater* **7**, 4131, 2011.
27. Zhang, R.Y., and Ma, P.X. Porous poly(L-lactic acid)/apatite composites created by biomimetic process. *J Biomed Mater Res* **45**, 285, 1999.
28. Fu, L.J., Li, J.X., Yang, X.G., and Wang, K. Gadolinium-promoted cell cycle progression with enhanced S-phase entry via activation of both ERK and PI3K signaling pathways in NIH 3T3 cells. *J Biol Inorg Chem* **14**, 219, 2009.
29. Zhang, Y., Fu, L.J., Li, J.X., Yang, X.G., Yang, X.D., and Wang, K. Gadolinium promoted proliferation and enhanced survival in human cervical carcinoma cells. *Biometals* **22**, 511, 2009.
30. Polini, A., Pisignano, D., Parodi, M., Quarto, R., and Scaglione, S. Osteoinduction of human mesenchymal stem cells by bio-active composite scaffolds without supplemental osteogenic growth factors. *PLoS One* **6**, e26211, 2011.
31. Kim, K., Dean, D., Lu, A., Mikos, A.G., and Fisher, J.P. Early osteogenic signal expression of rat bone marrow stromal cells is influenced by both hydroxyapatite nanoparticle content and initial cell seeding density in biodegradable nanocomposite scaffolds. *Acta Biomater* **7**, 1249, 2011.
32. Zhang, Y., Reddy, V.J., Wong, S.Y., Li, X., Su, B., Ramakrishna, S., and Lim, C.T. Enhanced biomineralization in osteoblasts on a novel electrospun biocomposite nanofibrous substrate of hydroxyapatite/collagen/chitosan. *Tissue Eng Part A* **16**, 1949, 2010.
33. Caravan, P. Strategies for increasing the sensitivity of gadolinium based MRI contrast agents. *Chem Soc Rev* **35**, 512, 2006.
34. Fralix, T.A., Ceckler, T.L., Wolff, S.D., Simon, S.A., and Balaban, R.S. Lipid bilayer and water proton magnetization transfer: effect of cholesterol. *Magn Reson Med* **18**, 214, 1991.
35. Washburn, N.R., Weir, M., Anderson, P., and Potter, K. Bone formation in polymeric scaffolds evaluated by proton magnetic resonance microscopy and X-ray microtomography. *J Biomed Mater Res* **69**, 738, 2004.
36. Xu, H., Othman, S.F., Hong, L., Peptan, I.A., and Magin, R.L. Magnetic resonance microscopy for monitoring osteogenesis in tissue engineered construct *in vitro*. *Phys Med Biol* **51**, 719, 2006.

Address correspondence to:
Shantikumar V. Nair, PhD

Amrita Centre for Nanosciences and Molecular Medicine
Amrita Institute of Medical Sciences and Research Centre
Amrita Vishwa Vidyapeetham University
Kochi 682041
India

E-mail: shantinair@aims.amrita.edu

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