The fabrication of nano-hydroxyapatite on PLGA and PLGA/collagen nanofibrous composite scaffolds and their effects in osteoblastic behavior for bone tissue engineering

Michelle Ngiam a, Susan Liao b,⁎, Avinash J. Patil c, Ziyuan Cheng b, Casey K. Chan b, S. Ramakrishna b

a National University of Singapore (NUS) Graduate School (NGS) for Integrative Sciences and Engineering, Centre for Life Sciences (CeLS) #05-01, 28 Medical Drive, 117456, Singapore
b National University of Singapore, 117576, Singapore
c School of Biosciences and Bioengineering, Indian Institute of Technology Bombay (IITB), Mumbai 400076, Maharashtra, India

⁎ Corresponding author. Healthcare and Energy Materials Lab, Department of Mechanical Engineering, Nanoscience and Nanotechnology Initiative (NUSNN), Faculty of Engineering, Block E3, #05-14, 2 Engineering Drive 3, National University of Singapore, 117576, Singapore. Fax: +65 67730339.
E-mail addresses: michelle.ngiam@nus.edu.sg (M. Ngiam), liaosusan@tsinghua.org.cn (S. Liao), avinashjpatil@gmail.com (A.J. Patil), liezyy@nus.edu.sg (Z. Cheng), doschanc@nus.edu.sg (C.K. Chan), seeram@nus.edu.sg (S. Ramakrishna).

Article info
Article history:
Received 27 December 2008
Revised 20 March 2009
Accepted 25 March 2009
Available online 7 April 2009
Edited by: B. Olsen

Keywords:
Osteoblast
Bone graft
Nanocomposite
Bone tissue engineering
Hydroxyapatite

Abstract
Bone is a nanocomposite consisting of two main components, nano-hydroxyapatite (n-HA) and Type I collagen (Col). The aim is to exploit the nano-scale functional and material characteristics of natural bone in order to modulate cellular functions for optimal bone repair in bone graft systems. Here, we present an effective and novel technique in obtaining n-HA in cogitate with native apatite on electrospun nanofibers within minutes without any pre-treatment. Using an alternate calcium and phosphate (Ca-P) solution dipping method, n-HA was formed on poly(lactide-co-glycolide) acid (PLGA) and blended PLGA/Col nanofibers. The presence of the functional groups of collagen significantly hastened n-HA deposition closed to nine-fold. The quantity of n-HA impinged upon the specific surface area, whereby mineralized PLGA/Col had a greater surface area than non-mineralized PLGA/Col, whereas n-HA did not significantly improve the specific surface area of mineralized PLGA compared to pure PLGA. The novelty of the process was that n-HA on PLGA had a positive modulation on early osteoblast capture (within minutes) compared to pure PLGA. Contrary, cell capture on mineralized PLGA/Col was comparable to pure PLGA/Col. Interestingly, although n-HA impeded proliferation during the culture period (days 1, 4 and 7), the cell functionality such as alkaline phosphatase (ALP) and protein expressions were ameliorated on mineralized nano HA impinged upon the specific surface, whereby mineralized PLGA/Col had a greater surface area than non-mineralized PLGA/Col, whereas n-HA did not significantly improve the specific surface area of mineralized PLGA compared to pure PLGA. The novelty of the process was that n-HA on PLGA had a positive modulation on early osteoblast capture (within minutes) compared to pure PLGA. Contrary, cell capture on mineralized PLGA/Col was comparable to pure PLGA/Col. Interestingly, although n-HA impeded proliferation during the culture period (days 1, 4 and 7), the cell functionality such as alkaline phosphatase (ALP) and protein expressions were ameliorated on mineralized nano HA impinged upon the specific surface, whereby mineralized PLGA/Col had a greater surface area than non-mineralized PLGA/Col, whereas n-HA did not significantly improve the specific surface area of mineralized PLGA compared to pure PLGA. The novelty of the process was that n-HA on PLGA had a positive modulation on early osteoblast capture (within minutes) compared to pure PLGA. Contrary, cell capture on mineralized PLGA/Col was comparable to pure PLGA/Col. Interestingly, although n-HA impeded proliferation during the culture period (days 1, 4 and 7), the cell functionality such as alkaline phosphatase (ALP) and protein expressions were ameliorated on mineralized nano HA impinged upon the specific surface, whereby mineralized PLGA/Col had a greater surface area than non-mineralized PLGA/Col, whereas n-HA did not significantly improve the specific surface area of mineralized PLGA compared to pure PLGA. The novelty of the process was that n-HA on PLGA had a positive modulation on early osteoblast capture (within minutes) compared to pure PLGA. Contrary, cell capture on mineralized PLGA/Col was comparable to pure PLGA/Col. Interestingly, although n-HA impeded proliferation during the culture period (days 1, 4 and 7), the cell functionality such as alkaline phosphatase (ALP) and protein expressions were ameliorated on mineralized nano

Introduction
Increasing affluence around the world has a direct and tangible impact of increasing the average lifespan of a population in developed countries. Yet, it brings forth a myriad of new challenges such as dealing with health issues of an aging population. In U.S. alone, it is estimated that the number of bone graft procedures performed annually is more than 500,000, with more than half of which dedicated to spinal fusion cases [1]. The current medical need is to address problems pertaining to traditional bone graft systems such as implant failure due to lack of tissue regeneration around the implant surface, resulting in poor bone remodeling and loosening of implants. In recent years, tissue engineering has revolutionized the direction of research for orthopaedic applications because of the success of nanotechnological advancements in creating nouveau fabrication techniques for nano-scale materials such as nanofibers and nanofibrous scaffolds. Indeed, many agree that by mimicking the structural properties of natural tissue, in this case, the subtleties of extracellular matrix (ECM) proteins, particularly collagen fibrils in bone, enhanced absorption of biomolecules such as vitronectin is achievable on nano-scale materials due to their high surface area-volume ratio [2]. This in turn, leads to a more favorable environment for cellular interactions (cell-to-cell and cell-material) to improve symbiosis.

In order to optimize the functional performance of bone graft materials, one must envisage the chemical and structure properties of native bone. Bone in itself encompasses mainly nano-hydroxyapatite (n-HA) and Type I collagen [3]. The collagenous nanofibrils are the main structural proteins, contributing up to 30% of the dry weight of bone and 90–95% of the organic, non-mineral component in bone
been considered as potential substrates for bone grafts. Some natural selection of material. Natural polymers and synthetic polymers have as phase separation, self-assembly and co-precipitation) because of its 

scaffolds, followed by SBF treatment[12] to introduce apatite on the 

differentiation marker was used to assess the cellular activity of 

mineralization were demonstrated on PCL/HA scaffolds[11]. Other 

osteopontin (OPN) which is 

surfaces of the scaffolds. Noticeably, osteopontin (OPN) which is 

biomineralization of n-HA was achieved by employing an alternate 
calcium phosphate (Ca–P) dipping method. We chose the electro-

nanocomposite systems[10]. It was shown that PCL- 

osteocalcin (OCN) and bone 

substrates, indicative of the in 

The aim of this study was to develop a novel biomineralized 

nanofibrous polymer scaffold via a biomimetic route. Various types of 

ease of fabrication to get various fiber patterns: random/aligned 

ease of scaling up is not a problem. For instance, polyglycolic acid 

metabolite and can be excreted out of the body as urine. Yet one of the 

because its degradable product, i.e. glycolic acid, which is a natural 

non-collagenous proteins for example, osteopontin, 

The paramountcy of the longevity of any biomaterial lies in the 

selection of material. Natural polymers and synthetic polymers have 

been considered as potential substrates for bone grafts. Some natural polymers investigated were collagen [5], chitosan [6], alginate [7] and silk [8] but the problem of using such natural materials is their inferiority in mechanical properties. To circumvent the drawbacks of natural polymers, biodegradable synthetic materials are attractive alternative options as they are generally mechanically better and the ease of scaling up is not a problem. For instance, polyglycolic acid (PGA) has been used medically as sutures and is often considered because its degradable product, i.e. glycolic acid, which is a natural metabolite and can be excreted out of the body as urine. Yet one of the disadvantages of using such materials is the release of acidic byproducts during degradation [9]. 

Often, a combination of ceramic-polymer materials is exploited to augment bone integration of the graft material and the native host tissue. Poly-γ-caprolactone (PCL)-grafted hydroxyapatite (HA) nanocrystals were used as an inorganic compound in the PCL/PCL- 

grafted HA nanocomposite systems [10]. It was shown that PCL- 

grafted HA resulted in better adhesion, proliferation of fibroblasts and protein adsorption compared to unmodified HA (non-PCL- 

grafted HA) and pure PCL controls. As the PCL-grafted HA content increased, improved cell anchorage and proliferation were obtained because more HA nanocrystals could be exposed on the nanocomposite surfaces, giving rise to enhanced protein pre-adsorption, cell adhesion and proliferation [10]. In a separate study, a precision extrusion deposition (PED) method was employed to prepare PCL and PCL/HA scaffolds. Alkaline phosphatase (ALP), an early bone differentiation marker was used to assess the cellular activity of osteoblasts. Clearly, improved osteoblast differentiation and matrix mineralization were demonstrated on PCL/HA scaffolds [11]. Other methods of incorporating HA into polymeric systems include the use of stimulated body fluids (SBF) [12,13], where the ionic concentration closely resembles that of human blood plasma. Solvent casting and particulate leaching process was employed to fabricate PLGA scaffolds, followed by SBF treatment [12] to introduce apatite on the surfaces of the scaffolds. Noticeably, osteopontin (OPN) which is another osteogenic differentiation marker, was significantly upregulated (up to 7-fold) on PLGA/HA scaffolds after 3 days. By week 4, late differentiation markers such as osteocalcin (OCN) and bone sialoprotein (BSP) were also significantly increased on the PLGA/HA substrates, indicative of the influence of HA in osteoblastic maturation [12]. 

The aim of this study was to develop a novel biomineralized nanofibrous polymer scaffold via a biomimetic route. Various types of nanofibers such as pure PLGA and blended PLGA/collagen (PLGA/Col) nanofibers were fabricated using an electrospinning technique and biomineralization of n-HA was achieved by employing an alternate calcium phosphate (Ca–P) dipping method. We chose the electrospinning method to prepare the nanofibers over other methods (such as phase separation, self-assembly and co-precipitation) because of its 

<table>
<thead>
<tr>
<th>Materials</th>
<th>Solvent</th>
<th>Solution concentration (w/v)</th>
<th>Feed rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Tip to collector distance (cm)</th>
<th>Humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA</td>
<td>HFP</td>
<td>20%</td>
<td>1</td>
<td>18</td>
<td>13</td>
<td>65</td>
</tr>
<tr>
<td>PLGA/Col</td>
<td>HFP</td>
<td>10%</td>
<td>1</td>
<td>18</td>
<td>13</td>
<td>65</td>
</tr>
<tr>
<td>[50:50]</td>
<td>HFP</td>
<td>6.66%</td>
<td>1</td>
<td>12</td>
<td>12</td>
<td>65</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Materials</th>
<th>Solvent</th>
<th>Solution concentration (w/v)</th>
<th>Feed rate (mL/h)</th>
<th>Voltage (kV)</th>
<th>Tip to collector distance (cm)</th>
<th>Humidity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen (Col)</td>
<td>HFP</td>
<td>6.66%</td>
<td>1</td>
<td>12</td>
<td>12</td>
<td>65</td>
</tr>
</tbody>
</table>

Fig. 1. Morphology of electrospun (a) pure PLGA and (b) PLGA/Col nanofibers.
voltage power supply from Gamma High Voltage Research, Florida, U.S.) was applied to draw the ultra-fine fibers from the spinneret (27G1/2 needle, Becton Dickinson, BD, NJ, U.S.) onto the collector plate. The spinneret was first grounded to give a flat tip in order to produce smooth and continuous nanofibers. A constant feed rate of 1 mL/h was adopted by means of a syringe pump (KD Scientific Inc., MA, U.S.). The electrospun nanofibers were subsequently vacuum dried so that any residual solvent present in the fibers could be removed.

**Mineralization of Nanofibers Using an Alternate Calcium–Phosphate (Ca–P) Dipping Method**

Mineralization of n-HA was achieved by subjecting the nanofibers in a series of calcium and phosphate treatments, deemed as the alternate dipping method [14]. PLGA and PLGA/Col nanofibrous scaffolds were first immersed in 0.5 M of CaCl₂ (pH of 7.2) (Aldrich Chemical Company, Inc., St. Louis, U.S.), followed by rinsing with de-ionised (DI) water. Next, the scaffolds were subsequently immersed in 0.3 M of Na₂HPO₄ (pH of 8.96) (Merck & Co. Inc., NJ, U.S.) and rinsed with DI water. The above-mentioned steps typified 1 cycle of Ca–P treatment. All nanofibers were subjected to 3 cycles of Ca–P treatments, where the first cycle was 10 min (in each chemical solution) and the second and third cycles were 5 min (in each chemical solution). After mineralization, the nanofibers were freeze-dried overnight. HA residues were collected in the DI water for X-ray diffraction (XRD) analyses.

**Material characterization**

The morphologies of both non-mineralized and mineralized nanofibers were analyzed using a field-emission scanning electron microscopy (FESEM) (Quanta 200F, FEI, Oregon, U.S.) at an...
accelerating voltage of 20 kV. The average fiber diameter of the scaffolds (n = 10 fibers) was determined from the FESEM micrographs using an image analysis software (Image J, National Institutes of Health, Bethesda, U.S.). In addition, the morphologies of the mineralized fibers were also studied using the Atomic Force Microscope (AFM) in tapping mode (Dimension 3100 AFM, Veeco Instruments Inc., CA, U.S.) using a silicon tip (Pointprobe-Plus silicon-SPM sensor, Nanosensors™, Switzerland) under ambient conditions with scanned areas of 1 x 1 μm. The images were recorded at a scan rate between 0.5 and 1 Hz. The cantilever tips had a tip radius of between 5 and 7 nm, with a resonance frequency of 315–369 kHz. The images were scanned at five predetermined areas for each material (n = 3). The crystallographic phases of the n-HA were analyzed using a powder X-ray diffraction (XRD) diffractometer (Shimadzu XRD 6000, Kyoto, Japan). All XRD measurements were carried out using Ni Filtered Cu Kα1 radiation, at a wavelength of 1.5406 Å with a scan rate of 2° min⁻¹ and a sampling interval of 0.02°. The 2θ range was fixed at 10–80°. The data was then analyzed using the XRD-6000 V2.5 software.

In order to determine the amount of HA present on the fibers, thermogravimetry analyses (TGA) (TA 2050 Thermogravimetric Analyzer, TA instruments, Tokyo, Japan) were done where the fibers (n = 5) were heated up to 1000 °C, at a heating rate of 10 °C/min in air conditions. The specific surface area of the substrates using the Brunauer-Emmett-Teller (BET) Analyzer (NOVA-3000 Ver 6.07, Quantachrome Instruments, Florida, U.S.) was studied as well. The fibers (n = 3–5) were degassed at room temperature for a day before measurements were taken.

**Osteoblast culture on nanofibrous scaffolds**

The nanofibers (round glass coverslips of 15 mm in diameter) were sterilized under ultraviolet (UV) light for an hour, followed by immersion in 70% ethanol for 10 min for sterilization purposes. The nanofibers were washed with phosphate buffered saline (PBS) thrice to eliminate any residual ethanol. Human fetal osteoblast cells hFOB 1.19 (American Type Culture Collection (ATCC), VA, U.S.) were cultured until passage 5 and seeded on the PLGA and blended PLGA/Col scaffolds at a cell concentration of 10⁴ cells/sample. The sample size for all material groups and tissue culture plastic (TCP) controls was three. The culture medium used consisted of the following constituents: 1:1 mixture of Ham’s F12 medium and
Dulbecco’s modified Eagle’s medium (DMEM) without phenol red with 2.5 mM L-glutamine (Invitrogen, CA, U.S.), 0.3 mg/ml G418 (Sigma Aldrich Chemical Company Inc., St. Louis, U.S.) and 10% fetal bovine serum (Invitrogen, CA, U.S). The medium was changed every two days to ensure that there was an adequate supply of nutrients present in the culture plate.

Cell adhesion study

1 mL of medium containing 10,000 osteoblasts was incorporated into individual wells containing the various types of nanofibers (non-mineralized and mineralized). The culture plates were subsequently incubated at different time points: 10, 20 and 30 min. After the incubation period, the media in each well was removed and the wells containing the samples were washed with PBS thrice. Methanol was added to the wells for 10 min at room temperature for cell fixation to take place. In order to determine the number of adhered cells on the material substrates, DAPI (4′, 6-diamidino-2-phenylindole, dilactate) (Invitrogen, CA, U.S) was utilized for staining the nuclei of the fixed cells (0.25 mL/well of 1 μg/mL DAPI) for 30 min at room temperature. Each test specimen was photographed using a fluorescence microscope (Leica DM IRB, Leica Microsystems, GmbH, Wetzlar, Germany) at 100× magnification at five predetermined regions (top, centre, bottom, left and right areas of the culture well) to arbitrate the number of attached fluorescence cells (nuclei count). This cell count method was also used to determine the number of attached cells at day 1, day 4 and day 7 of culture for cell proliferation quantifications.

Cell morphology

The nanofibrous scaffolds which were inoculated with osteoblasts (day 1, day 4 and day 7 of culture) were fixed in 4% paraformaldehyde (Lancaster, England) solution for 30 min at room temperature. Next, the scaffolds were dehydrated in a series of 5 cycles (1 cycle: 10 min) with increasing concentrations of ethanol: 50%, 70%, 95%, and 100% (twice) respectively. Finally, the samples were air-dried overnight and SEM imaging was done to view the morphology of the attached cells on the scaffolds.

Total protein assay

A cell concentration of 10,000 cells was seeded in each well containing the substrates and tissue culture plate (TCP) controls and cultured for 1, 4, and 7 days. At the end of the incubation periods, the medium was removed from the culture wells and washed with PBS.
thrice. A bicinchoninic acid (BCA) protein assay working reagent was used (BCA Kit No.23225, Pierce Biotechnology, IL, U.S.) and the culture plates were incubated for 30 min at 37 °C. The protein content was obtained by using a FLUO Star Optima microplate reader (BMG Labtech GmbH, Offenburg, Germany), at a wavelength of 590 nm at room temperature. Employing a standard curve of absorbance versus known concentrations of albumin (from the BCA kit), the total intracellular protein (expressed in milligrams) synthesized by the osteoblasts was acquired. Protein concentration results were normalized by the cell numbers.

Alkaline phosphatase (ALP) expression

ALP activity was measured at all time points (days 1, 4 and 7). ALP expression was normalized by the cell number at each culture time point. Briefly, 400 μL of p-Nitrophenyl Phosphate, Disodium Salt (PNPP) solution (Phosphatase Substrate Kit, No. 37620, Pierce Biotechnology, IL, U.S.) was added to each well and incubated at room temperature for an hour. This was followed by adding 200 μL of 2M NaOH to terminate the reaction of p-nitrophenol conversion to p-nitrophenylate. A FLUO Star Optima microplate reader (BMG Labtech GmbH, Offenburg, Germany) was used to measure the amount of light absorbance, at a wavelength of 405 nm.

Mechanical testing of nanoﬁbrous scaffolds

Mechanical testing of the nanoﬁbrous scaffolds (with or without cells) was done. The culture period was stipulated at 1, 4 and 7 days. After the incubation periods, the cell/scaffold constructs were freeze-dried overnight at −60 °C. The nanoﬁbrous scaffolds were detached from the aluminium foil, using a paper frame with double-sided tape attached to it. The purposes of the frame were to provide additional support to the sample ends, for the fixation in the tensile tester clamps and to ensure a standardized and constant test size for all the samples to be analyzed (10 mm width, 20 mm length). The thickness of the nanoﬁbrous membrane was determined by means of a micrometer (Mitutoyo America Corporation, IL, U.S.) and the average thickness was calculated by taking the average of the measurements at three positions. The average thickness value was then used to calculate the cross-sectional area of the specimen. The sample size for each material type was ﬁve. Each specimen was tested using an Instron Machine (Instron Micro Tensile Tester 5848, MA, U.S.) and subjected to 2.5 Newton Load. The deforming speed was 5 mm/min and the sampling rate was 100 Hz. The E-modulus was calculated using the 40 data points obtained in the linear region of the deformation curve.

\[
\sigma = \frac{F}{A}
\]

\(\sigma\): stress (Pa), \(F\): force (N), \(A\): Cross-sectional area (m²).

\[
\varepsilon = \frac{L_{\text{change}}}{L}
\]

\(\varepsilon\): strain, \(L_{\text{change}}\): change in length (m), \(L\): original length (m)

\[
E = \frac{\sigma}{\varepsilon}
\]

E: E-modulus (Pa), \(\sigma\): stress (Pa), \(\varepsilon\): strain.

Fig. 4. Hydrophilicity property of nanofibers. Significant difference between various material groups were denoted as * (p< 0.05).

Fig. 5. X-ray diffraction (XRD) results of, (a) mineralized PLGA + n-HA nanofibers, (b) mineralized PLGA/Col + n-HA nanofibers and (c) natural tooth (control).

Fig. 6. Fourier transform infrared (FTIR) spectra of (a) PLGA (b) Collagen (Col) (c) PLGA/Col, (d) mineralized PLGA + n-HA and (e) mineralized PLGA/Col + n-HA nanofibers. Peaks at 1761 cm⁻¹ and 1088 cm⁻¹ were assigned to the carbonyl and C–O stretch respectively in (a) PLGA. Amide I and II groups of collagen were detected at peaks 1658 cm⁻¹ and 1544 cm⁻¹ respectively. Peaks at 1039 cm⁻¹ and 588 cm⁻¹ represented the phosphate groups and peaks at 1631 cm⁻¹ and 866 cm⁻¹ were referred to the carbonate groups from n-HA in (d) PLGA + n-HA and (e) PLGA/Col + n-HA.
Statistical analysis

The data collected was expressed as mean ± standard deviation (S.D.). The Student’s T-test (T-test) was also employed to determine the differences between the various material groups. A p-value of less than 0.05 (p < 0.05) was considered to be of significant difference.

Results

Material characterization of nanofibrous scaffolds

The morphology of the PLGA and PLGA/Col nanofibers were smooth and of relatively uniform thickness based on the electropinning parameters we employed as seen in Fig. 1. The average diameters of the PLGA and PLGA/Col nanofibers were 598 ± 65 nm and 353 ± 81 nm respectively. Mineralization of n-HA was successfully deposited on pure PLGA and PLGA/Col nanofibers after three rounds of Ca–P treatment as depicted in Fig. 2. As shown in Figs. 2(d)–(f), n-HA was formed homogenously on PLGA/Col nanofibers, unlike those formed on pure PLGA substrates (Figs. 2(a)–(c)). As evident in the high resolution SEM micrographs (Figs. 2(c) and (f)), n-HA formed on PLGA and PLGA/Col nanofibrous scaffolds were nanocrystalline in structure. For PLGA nanofibers, n-HA deposition was predominately on the surfaces of the fibers, whereas n-HA deposition occurred throughout the PLGA/Col nanofibrous scaffolds (both exterior and interior). AFM images showed some form of nanotexturing of the fibers, when n-HA was incorporated on the fibers as seen in Fig. 3. From Figs. 3(e) and (f), the n-HA size was approximately 50–70 nm in diameter for both mineralized PLGA and PLGA/Col. Representative 3D renderings of the mineralized fibers indicated that the surface topography was dissimilar: the incorporation of collagen in PLGA (Fig. 3h-PLGA/Col + n-HA) resulted in spiky ridges and grooves on the surfaces, whereas mineralized PLGA (Fig. 3g-PLGA + n-HA) showed a somewhat plateau surface with some non-spiky ridges and grooves.

Fig. 7. Human fetal osteoblasts (hFOB) capture on mineralized and non-mineralized PLGA and PLGA/Col nanofibers at 10 min, 20 min and 30 min time points at room temperature. Significant difference between different material groups were denoted as * (p < 0.05).

Fig. 8. Cell proliferation on mineralized and non-mineralized PLGA and PLGA/Col nanofibers after 1, 4 and 7 days of culture. Significant difference between different material groups were denoted as * (p < 0.05).
From Fig. 4, it was observed that the presence of n-HA on PLGA (107.5° ± 1) significantly increased the hydrophobicity of pure PLGA (75.9° ± 4.9) nanofibers. Conversely, by blending collagen with PLGA (29.1° ± 3.4), n-HA deposition significantly mellowed the hydrophilicity of the PLGA/Col + n-HA (11.2° ± 2.7) scaffolds, to a greater degree in comparison with pure collagen nanofibers (54.6° ± 8.7).

Our XRD results in Fig. 5 delineated the presence of n-HA in all the mineralized nanofibrous scaffolds (Fig. 5a: PLGA + n-HA and Fig. 5b: PLGA/Col + n-HA). All the peaks in Figs. 5a and b were in cognate with the peaks associated with natural tooth (Fig. 5c), suggestive that our method of rapid mineralization was effective in producing n-HA phases (Ca–P ratio = 1.67).

FTIR spectrum was carried out to confirm the functional groups of the treated nanofibrous scaffolds. Our observations showed that both phosphate and carbonate groups were present in mineralized PLGA (Fig. 6d) and PLGA/Col nanofibers (Fig. 6e). In pure PLGA nanofibers (Fig. 6a), the C=O stretch and the C–O stretch hovered around 1761 and 1088 cm\(^{-1}\) respectively. Amide I and amide II of collagen were detected at 1658 cm\(^{-1}\) and 1544 cm\(^{-1}\) in PLGA/Col (Fig. 6c) substrates. The wavenumbers for phosphate groups were characterized at approximately 588 cm\(^{-1}\) and 1039 cm\(^{-1}\). For carbonate groups, the peaks were seen at around 866 cm\(^{-1}\) and 1631 cm\(^{-1}\). Particularly, there was more carbonated component of Fig. 6(e) mineralized PLGA/Col + n-HA than Fig. 6(d) mineralized PLGA + n-

![Fig. 9. Osteoblasts on mineralized and non-mineralized nanofibers. (a) PLGA (day 1), (b) PLGA + n-HA (day 1), (c) PLGA + n-HA (day 4), (d) is the enlargement of the central part of (c), (e) PLGA/Col (day 1), (f) PLGA/Col + n-HA (day 1), (g) PLGA/Col + n-HA (day 4).](image-url)
HA, owing to the stronger peak signal at 866 cm\(^{-1}\). The N–H bend of amide I corresponding to 1658 cm\(^{-1}\) and the N–H bend of amide II peaked at 1544 cm\(^{-1}\) which was seen in Fig. 6(c) PLGA/Col and Fig. 6(e) PLGA/Col + n-HA, although these peaks overlapped with the peak (1631 cm\(^{-1}\)) corresponding to the carbonate group in PLGA/Col + n-HA. Furthermore, despite that the absorption band (1088 cm\(^{-1}\)) of the C–O stretch from PLGA (Fig. 6a) was overlapping with the one of the bands (1039 cm\(^{-1}\)) of the phosphate group in n-HA, the relative intensities of the phosphate groups were more prominent in mineralized Fig. 6(d) PLGA+n-HA and Fig. 6(e) PLGA/Col+n-HA.

From our TGA results, the amount of n-HA in mineralized PLGA and PLGA/Col were 8.3%±6.1 and 73.9%±10.7 respectively. Furthermore, the average specific surface area for mineralized PLGA+n-HA (4.5±1.9 m\(^2\)/g) nanofibers was not significantly different than pure PLGA nanofibers (3±1.3 m\(^2\)/g). Not surprisingly, the specific surface area of PLGA/Col+n-HA (25.9±4.1 m\(^2\)/g) was significantly higher than PLGA/Col (15.2±3.5 m\(^2\)/g) nanofibers, which was in line with our TGA results, where the mineralized PLGA/Col constituted a larger amount of n-HA, thereby increasing the surface area of the fibers.

Enhanced cell capture on mineralized and collagen blended nanofibrous scaffolds

As depicted in Fig. 7, mineralized PLGA (PLGA + n-HA) significantly increased the initial cell number as compared to pure PLGA nanofibers at all time points (10, 20 and 30 min.). Contrary, there was no enhancement of cell capture on mineralized PLGA/Col (PLGA/Col + n-HA) compared to PLGA/Col substrates at all time points, suggesting that there was no synergistic effect between collagen and n-HA for early cell capture. Concurrently, the combined results of this study showed not only the effect of n-HA on early cell attachment but also blended collagen alone was able to enhance cell capture. In general, PLGA/Col, mineralized PLGA and PLGA/Col fared better than TCP controls throughout this cell capture experiment.

Cell behavior on nanofibrous and mineralized nanofibrous scaffolds

In Fig. 8, it was noted that the cell number for mineralized PLGA and PLGA/Col were comparable at all time points. When compared
with the non-mineralized PLGA nanofibers, there was no significant difference in cell number for PLGA+n-HA after day 1 but the cell number tapered off after days 4 and 7 on mineralized PLGA fibers. Similarly, there was a significant reduction of cells on the mineralized PLGA/Col nanofibers as compared to PLGA/Col nanofibers from days 1 to 7. From days 4 to 7 of culture, the cell numbers leveled off on pure PLGA, mineralized PLGA+n-HA and PLGA/Col+n-HA when compared to our TCP and collagen controls. At all culture periods, there was an enhancement of cells on pure PLGA nanofibers. Likewise, blended collagen PLGA and pure collagen nanofibers had a significant improvement in the cell number over the culture periods.

As evident in the SEM micrographs, osteoblasts were successfully seeded on both mineralized and non-mineralized nanofibers, where the cells adhered to both n-HA and non n-HA regions of the nanofibers. After day 1 of culture, cell spreading was more apparent on PLGA + n-HA (Fig. 9(a)) as compared to those grown on pure PLGA nanofibers (Fig. 9(a)). At day 4, the cells were strongly anchored on the PLGA + n-HA nanofibrous scaffolds, with preferential attachment of the pseudopodia to n-HA regions as seen in Figs. 9(c) and (d). In addition, it was observed that there was a greater propensity of cell spreading on mineralized PLGA/Col nanofibers (Figs. 9(f) and (g)) compared to mineralized PLGA nanofibers (Figs. 9 (b)−(d)) at specified cell culture time point, with osteoblasts enshrouding most of the nanofiber surfaces at day 4 with extended lamellipodia, creating a cell monolayer on the fibers (Fig. 4g).

Fig. 10 illustrates the total protein concentration of the cells at various culture periods. Protein concentrations for PLGA + n-HA increased after days 4 and 7 compared to pure PLGA substrates. The presence of n-HA on PLGA/Col led to an increase in protein secretion at all culture time points compared to PLGA/Col nanofibers. In general, mineralized PLGA and PLGA/Col were comparable throughout the culture period. Coincidentally, mineralized PLGA and PLGA/Col fared better than pure collagen substrates and TCP controls, giving good evidence that n-HA enhanced protein secretion of the osteoblasts.

ALP, a bone marker, was measured and shown in Fig. 11. There was a slight reduction in ALP expression on the PLGA + n-HA nanofibers than pure PLGA nanofibers after day 1 of culture, yet there was a significant increase in ALP on PLGA + n-HA on days 4 and 7 when compared to its pure PLGA counterparts. The incorporation of n-HA on PLGA/Col fibers had prevailed inclusively being statistically higher than non-mineralized PLGA/Col fibers from days 1 to 7. Results of ALP expression of mineralized PLGA and PLGA/Col coincided on early culture time points (days 1 and 4), but on day 7, ALP expression was mitigated in mineralized PLGA/Col than mineralized PLGA substrates. One noteworthy observation made was that ALP expression in mineralized PLGA and PLGA/Col surpassed those of collagen and TCP controls and pure PLGA/Col were comparable to collagen throughout this study.

The stiffness (E-modulus) of the material constructs namely PLGA and mineralized PLGA nanofibers were significantly enhanced after day 1 of culture, as compared to those without cells as seen in Fig. 12. Despite a longer culture period at day 4, no significant improvement in mechanical property was exhibited in all the material groups. The E-modulus between pure PLGA and mineralized PLGA were similar except for a dip in mechanical strength of PLGA + n-HA compared to pure PLGA after 1 day of culture. Mineralized PLGA/Col nanofibers did not have an increase in stiffness at days 1 and 4 of culture. Results for day 7 were not obtainable because of sample damage owing to the excessive tackiness of the fibers, possibly due to enhanced expression of extracellular matrix (ECM) proteins after prolonged culture periods.

Discussion

An emerging area of interest in bone tissue engineering is to develop processes and design materials which can be effectively resemble the nanostructure of native bone. Principally, synthetic polymers are utilized in attempt to mimic the main ECM protein in bone, type I collagen. Owing to the composition of bone, HA is often incorporated in material substrates via various modes of processing methods. In our study, we have successfully deposited bone-like nano-apatite on polymeric electrospun PLGA and PLGA/Col nanofibers using a biomimetic Ca-P treatment, without the need for pre-treatment such as alkaline treatment in order to hydrulize the surfaces for initial apatite nucleation. The presence of collagen in the nanofibers expedited the deposition of n-HA on the scaffolds. It was emblematic of the types of functional groups present in collagen, i.e. scillect carboxyl groups and carbohydrate groups [15,16]. These functional groups served as nucleation sites for apatite formation and consequently, uniform distribution of n-HA was apparent on the outer and inner surfaces of the PLGA/Col nanofibers. Besides being a favorable site for nucleation, the −COOH functional groups of collagen augmented the hydrophilicity property of the nanofibers, unlike for pure PLGA nanofibers, the addition of n-HA increased the hydrophobicity of the scaffolds. Lastly, the extent of n-HA formation was more evident in PLGA/Col nanofibers as compared to PLGA + n-HA based on our SEM, TGA and AFM results. Our investigations showed that n-HA were present in all mineralized PLGA substrates, where the broadening and overlapping of peaks in our mineralized substrates were attributed to the minute fine grain size of the crystal, where it was lower than natural human teeth. Phosphate bands in HA were observed in our FTIR spectrum, located at positions 1039 cm<sup>−1</sup> and 588 cm<sup>−1</sup>, which were associated with the molecular vibrations of phosphate. Collectively, the XRD and FTIR results were in good attainment with our recent work where the mineralization method was employed on PLLA and PLLA/Col nanofibers [17]. The motivation for studying both PLLA and PLGA systems was to investigate whether the type of material used impinged upon mineralization. In addition, the material degradation properties for bone applications rendered consideration and PLGA could be varied by manipulating the LA and GA ratio.

![Fig. 12. E-modulus (in MPa) values of various PLGA substrates after 1 and 4 days of culture. Significant difference between different material groups were denoted as * (p<0.05).](image-url)
Our TGA results clearly demonstrated that although the treatment cycle and duration period were akin for both PLGA and PLGA/Col, there was a large diminution in the quantity of n-HA on the PLGA substrates, further corroborating that collagen served as an excellent template for n-HA mineralization. Another possible could also be due to the larger fiber diameter of PLGA (598 ± 65 nm) than PLGA/Co (353 ± 81 nm), thereby the smaller diameter PLGA/Col provided more surface area for n-HA deposition. Taken together, our previous work showed that for PLGA/n-HA (5 cycles—10 min/solution) and PLGA/Col/n-HA (3 cycles—10 min/solution), the quantity of n-HA formed on these PLGA substrates were somewhat similar (PLLA + n-HA: 37.8 wt%, PLGA/Col + n-HA: 30.2 wt%), indicative that the presence of collagen had a greater effect on n-HA deposition, despite lesser treatment cycles for PLLA/Col substrates [17]. Contrary to what was observed for mineralized PLLA substrates [17], there was approximately 2.4 times more n-HA was found on PLGA/Col + n-HA than PLLA/Col + n-HA although the treatment cycles were shorter for PLGA/Col + n-HA substrates. One possible explanation could be that there were more PLGA/Col nanofibers (depending on the duration of fiber collection during electrospinning) subjected to Ca–P treatment, thus providing greater surface area for n-HA deposition. Compared to PLLA, the quantity of n-HA on pure PLGA substrates was markedly lower, mainly because of the shorter duration of treatment and number of cycles. As expected, the presence of n-HA on the fibers increased the specific surface area on mineralized PLGA/Col. For instance, electrospun PLLA/HA (12.44 m²/g) saw an increase in specific surface area compared to pure PLLA fibers (3.33 m²/g) [18]. In our case, although less n-HA was found on mineralized PLGA (PLGA + n-HA) compared to mineralized PLGA/Col (PLGA/Col + n-HA), the specific surface area was comparable between PLGA + n-HA and non-mineralized PLGA, indicating that besides the nanostructural topography of the n-HA, the quantity of n-HA had an influence on the specific surface area of the substrates.

The rationale for conducting a short-term cell attachment on the nanofibrous scaffolds was to assess the viability of avoiding extended culture periods of cell seeding on the substrates, thereby reducing the down-time from material preparation to the material implantation in the patient, preferably in-situ during surgery. Early cell adhesion on surfaces may not necessarily translate into cell spreading behavior, and good cell attachment at early time points may not impinge upon desirable cell proliferation and differentiation [19,20]. Indeed, n-HA significantly enhanced the initial cell number for mineralized PLGA, but this phenomena was not evident on mineralized PLGA/Col substrates. Our previous work demonstrated that both mineralized PLLA and PLGA/Col had an enhancement of cells at early time points, as compared to their non-mineralized counterparts where the presence of collagen and n-HA had a synergistic effect for early cell capture [17]. In this respect, the quantity of n-HA present on mineralized PLLA (37.8 wt%) and PLGA/Col (30.2 wt%) was comparable. These responses, in turn, may be ascribed to this plausible explanation—that an optimal quantity of n-HA may be needed for early cell attachment and excessive quantities may be detrimental to cell attachment, whereas PLGA/Col + n-HA had more n-HA than PLLA/Col + n-HA.

In a separate study, the nanotopography of HA and β-tricalcium phosphate (β-TCP) did not have an effect on cellular attachment (cell spreading, focal contacts and stress fiber formation) [21]. There was good evidence which indicated that material chemistry and grain size and not surface roughness and crystal phase had an effect on cell attachment of rat osteoblasts, fibroblasts and human mesenchymal stem cells (hMSCs) [22]. Clearly, such observations led to the difficulty of dissecting the true mechanism of osteoblastic behavior, but rather a network of material properties such as surface chemistry, surface energy, surface topography and so on, was likely at work. Concurrently, enhanced early cell capture was clearly seen for mineralized substrates over TCP controls, where the nano-scale character of these fibers were exhausted to its full potential in promoting cell attachment, contrarily to what was observed for TCP controls and micron-sized (2–5 μm) HA particles [23] whereby they shared the same superiority in cell attachment at 90 min over other materials such as CoCr, Ti, TiN and so on. In addition, there is evidence [16,24,25] that n-HA attracts osteoblasts substantially, thus this is a driving motivator for us to develop such biomimetic n-HA based materials. In the light of the fact that the surface topography of PLGA + n-HA and PLGA/Col + n-HA was different, one may reason that certain features of the physical environment could actually have a negative impact on initial cell attachment. For instance, certain stress mediators such as p38 and c-Jun N-terminal kinase (JNK) were significantly activated in 3D calcium phosphate scaffolds [26], thereby indicating that cells response to environmental signals, triggering certain signaling pathways such as mitogen-activated protein kinase (MAPK) cascade. Such stress-signaling response was less evident in 2D calcium phosphate scaffolds [26]. Again, this highlights the complexity of cellular response on various material types and such differences are not notorious as these can be attributed to a multi-factorial system.

During the culture period, n-HA attenuated the number of cells on mineralized PLGA and PLGA/Col nanofibers, even though the nanotopography of mineralized PLGA and PLGA/Col were dissimilar. This was in agreement with our previous study where mineralized PLLA and PLGA/Col had a significant cell reduction as compared to their non-mineralized counterparts [17], contrarily to what was observed in other studies where osteoblast adhesion was improved on nanophase HA materials [24,27]. Coincidentally, previous work had also shown that the surface roughness and crystal phases did not influence the proliferation of osteoblasts, fibroblasts and human mesenchymal stem cells (hMSCs) [22]. Likewise, as previously mentioned, n-HA increased the hydrophobicity of the PLGA nanofibers but this was not concordance to the observation obtained for mineralized PLGA/Col nanofibers whereby the hydrophilicity was increased, most likely due to the hydroxyl (–OH) groups of collagen attributing to greater wettability and surface polarity [28]. Not surprisingly, for pure collagen and PLGA/Col nanofibers, there was a significant elevation in the number of cells at all culture time points, where hydroxyl groups promoted cell adhesion and growth. From this, we can infer that collagen alone may seem to be a more favorable material for the increment of cells during culture, judging from the proliferative capability of the cells alone. Besides, the “smooth-texturing” of our non-mineralized nanofibers which led to greater adhesion of bone-like cells was also observed in a separate study, where increased adhesion and growth of osteoblast-like cells and smooth muscle cells were prevalent on polished surfaces [29]. Contrarily to what was observed that cell adhesion was elevated on HA surfaces, some studies also showed that there was no significant difference in cell number between PCL and PCL/HA scaffolds [11]. They postulated that the cells could have migrated into the inner depths of the scaffolds and a possibility of a lack of nutrients within the scaffolds [11]. Likewise, n-HA coatings were shown to decrease osteoblast-like cells and could be due to the negative cues ascribed by curved HA surface at a nanometer level [30]. An extensive myriad of published results [29,31,32] was akin to those observed in our study, where surface topography had an influence on cell behavior, which was accompanied by attenuated proliferation rates on rougher surfaces.

Besides the quantification of the number of cells present on the nanofibrous scaffolds, cellular behavior is another pivotal indicator to determine the potential application of a material construct for any tissue engineering application. Extensive cell spreading was obvious on mineralized PLGA/Col nanofibers compared to PLGA/HA nanofibers. Such observation was in concordance to those seen in mineralized PLLA and PLGA/Col nanofibers [17]. Likewise, cell spreading, with spindle-like and polygonal-like cell shapes, was also observed on HA-based composites after 3 days of culture and physical
contact between cells were maintained via filopodia or lamellipodia [33]. Should the culture period be prolonged in our study, it would not be surprising to obtain full cell coverage on the nanofibers as seen in the study by Zhang et al. where multi-layers of osteoblasts overlaying almost the entire surface of the electrospun HA/chitosan nanofibers after 10 to 15 days of culture, whereby ECM secreted by the osteoblasts and apatite-like mineral deposits were also observed [34]. It is widely known that surface topography affects the cellular behavior. Minute voids in n-HA, coupled with specific ECM protein absorptions and peptide (e.g. Arg–Gly–Asp or RGD in short) tethering on n-HA may have provided signals for the development of filopodia and lamellipodia of the osteoblast cells [30], although these preliminary results render further investigations. Superior cell spreading was seen especially on mineralized PLGA/Col and it was ascribed to the fact collagen contained cell-adhesion domain sequences such as RGD, whereby collagen was able to bind to the integrins α1β1 and α2β1, enhancing cellular interactions of osteoblasts and the fiber surfaces via integrins [35]. Although n-HA decreased the cell number, n-HA may have a chemoattractant role for filopodia formation due to selective protein absorptions. By virtue of these observations, n-HA or n-HA in combination with collagen would result in greater cell motility due to better-developed filopodia and lamellipodia [30]. Despite the fact that the number of cells adhered to mineralized PLGA/Col nanofibers was lower than those without n-HA (pure PLGA and PLGA/Col), from the SEM images, HA nanocrystallites formed on the PLGA/Col nanofibers may act as anchoring sites for the recruitment of bone-like cells in in-vivo situations.

One of the properties of HA is its bioactive nature which promotes osteoblastic differentiation in vitro [36–38]. ALP is an early osteogenic marker for differentiation that is important for the construction of bone matrix. In addition, we considered the protein secretion levels (protein expressions) as an epiphemomenon of cell viability, after the initial events of cell adhesion and proliferation. Taking into account of the results in this study, it was cogent that n-HA had a positive influence on ALP and protein expressions. Contrary to the promotion of ALP expression on our mineralized PLGA and PLGA/Col nanofibers, the differences in ALP expression between mineralized PLLA and PLLA/Col were comparable [17]. This again may be due to the different HA quantities on the mineralized PLLA and PLGA substrates. In general, our results pertaining to this study was in good agreement with Kim et al.’s study whereby there was enhancement of ALP activity on the PLGA/HA composite scaffolds [39]. There was general consensus of our results with another study, in which HA steadily increased ALP activity in PCL scaffolds from days 7 to 21 [11]. Similarly, apatite-coated PLGA/HA had greater ALP levels than non-apatite-coated PLGA/HA scaffolds and this was seen after two weeks and ALP levels between these two materials were comparable after 7 days [13]. Furthermore, enhanced cell differentiation and impaired proliferation were also observed on HA surfaces [21]. In addition, hMSCs cultured on mineralized silk fibroin scaffolds showed enhanced osteoconductive characteristics, accompanied by the presence of HA and bone morphogenetic protein-2 (BMP-2) [40]. In this circumstance, our results showed that even after day 1 of culture, the ALP expression was readily detectable on mineralized PLGA/Col nanofibers, where an increase was seen, highlighting the fact that cells were not only responsive to surface chemistry of the material, but were also able to discriminate minute variations in topography and surface energy.

The protein secretions by the osteoblasts on mineralized PLGA and PLGA/Col were significantly higher than the non-mineralized PLGA and PLGA/Col substrates respectively, correlating with our previous work [17] on mineralized PLLA and PLLA/Col, possibly due to the greater surface area-to-volume ratio of mineralized substrates. The results in the increase of protein expression were corroborated with several other studies [41,42]. In a separate study, protein expression on TCP controls was either comparable to HA reinforced starch-based polymers at early time points (day 1 and day 3) or significantly higher at a later time point (day 7) [43]. It is noteworthy to highlight that the ALP and protein expressions had been normalized to the respective cell numbers according to culture time point, thus suggestive that although the starting number of cells present on mineralized substrates was far lesser than non-mineralized materials, there was greater cellular activity per osteoblast on mineralized fibers. Likewise, the presence of collagen failed to elicit a favorable response as depicted by the less-than-optimal cell performance in terms of ALP expression and protein secretion in blended PLGA/Col compared to pure PLGA nanofibers, suggestive that n-HA alone had positive influence on cellular behavior.

For any tissue-engineered construct to be a functional implant, it is imperative to determine the material properties and compare against that of natural bone. The Young’s Modulus or E-modulus of cortical bone and cancellous bone is between 12.8 and 17.7 GPa and 0.4 GPa respectively [44]. In our study, the E-modulus of pure PLGA and mineralized PLGA nanofibers were significantly ameliorated after 1 day of culture, compared to those materials without cells. Only mineralized PLLA showed an improvement in E-modulus from days 1 and 4 of culture [17]. Indeed, the inclusion of n-HA in composite scaffolds showed an improvement in mechanical properties [42,45]. The stiffness of mineralized PLGA/Col nanofibers was comparable at day 1 and day 4 of culture. We speculated that the collagen in the nanofibers had undergone degradation during culture and water absorption could also be a contributing factor to the above-mentioned observation. Since the amount of ECM proteins which were secreted by cells could be limited at early culture periods, the presence of ECM proteins may be incommensurate to counter the effects of the degradation of the collagen. The E-modulus of our mineralized substrates without cells hovered around 50 MPa. On the other hand, the E-modulus of PLGA/HA scaffolds which were chemically synthesized containing different HA concentrations ranged between 2.3 and 4.6 MPa, where higher HA inclusion (30 wt.%) impaired the E-modulus compared to one with a lower HA content (10 wt.%) [46]. Several factors for the disparity of these results could be due to structural or lattice defects of HA, weak adhesion of HA at the polymer interface or porosity at HA–polymer interface and so on [46]. Despite varying mechanical properties of different HA-based materials, there is a general consensus that it is an onerous obstacle to match the true mechanical values of native bone.

The preliminary results render further investigations as the conceptual approach of employing n-HA based constructs is not fully lucid, given the extensive plexus of biological pathways, gene and protein expressions associated with mineralization and bone cells. Long-term cell culture studies and late osteogenic markers are imperative to assess the full potential of such systems. The general paradigm of these approaches in designing bone graft systems focuses on augmenting cell differentiation towards an osteoblastic lineage and escalation in bone remodeling at the site of implantation. Since our culture period was only up to 7 days, the results obtained may only be a snapshot of the initial cell behavior. One possible improvement in the future may involve the study of calcium deposition of the cultured osteoblasts over a longer period since mineralization takes place in a continuous manner during culture. This in-situ method of producing n-HA on polymeric nanofibers may be a probable option for future bone graft materials.

Conclusion

Bone-like apatite was formed on PLGA and PLGA/Col nanofibrous scaffolds by a bioimimetic Ca–P method. The preferential deposition of the n-HA crystallites was predominately on PLGA/Col nanofibers compared to PLGA nanofibers, suggesting that collagen was a good material template for n-HA nucleation. Mineralization mediated early cell capture on the fibers within a short span of time (within minutes). The functional activities (ALP and total protein expressions)
of the cells improved on mineralized nano-PLGA and PLGA/Col were comparable during the culture period. Furthermore, the presence of osteoblasts significantly elevated the stiffness of the material constructs. Our study showed that more attention had to be devoted towards understanding the interplay between material characteristics and cellular behavior for the optimization in designing better graft systems.

Acknowledgments

The first author would like to thank Mr. He Liium for his kind assistance in mentoring her in the use of the AFM instrument. This study was funded by NMRC/1151/2008, FRC-R397000036112 and LKY-PDF, National University of Singapore, Singapore.

References
