Directed Calcium Deposition by Osteoblasts Along Aligned Carbon Nanofiber Patterns on Polymers

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INTRODUCTION

Carbon nanotubes/nanofibers have many advantages in tissue engineering, especially in orthopedics[1-2]. This is because they are light-weight, strong and, possibly more important, can mimic the nanometer structures of components of bone (such as hydroxyapatite and collagen). Bone has also been shown to regenerative under electrical stimuli, thus conductive nanophase materials (like carbon nanotubes) may play a role in promoting osteoblast (bone-forming cells) activity important for orthopedic applications[3]. Previous studies have shown increased adhesion[1-2], viability[4] and deposition of calcium[5] by osteoblasts when cultured on carbon nanotube/nanofiber based materials. However, in all of these studies, non-aligned carbon nanotubes/nanofibers in polymers were investigated. Since long bones in the body are highly anisotropic[6], the objective of this in vitro study was to align micro-patterns of carbon nanofibers in a polymer matrix and determine subsequent osteoblast functions on these constructs.

EXPERIMENTALS

Carbon Nanofibers (CNFs)

Carbon nanofibers produced by carbon vapor deposition were obtained from Applied Sciences, Inc. (Cedarville, OH). These carbon nanofibers have a polynuclear aromatic hydrocarbon (PAH) layer, which is usually called a pyrolytic layer, formed during the production process[7]. Besides hydrophobic properties, a pyrolytic insulating outer layer has a lower surface energy (approximately 25mJ/m^2) compared to pyrolytic-free carbon nanofibers (approximately 100mJ/m^2). Only carbon nanofibers with a pyrolytic outer layer were used in the present experiments. The diameter of each carbon nanofiber (CNF) used was 150nm.

Polycarbonate Urethane (PCU)

An FDA-approved polycarbonate urethane (PCU, catalog # PC-3575A, Thermedics, MA) was used as the model polymer in this study since it has a high melting temperature (above 200˚C), is FDA approved for implantation, and is non-degradable.

Micro-Patterned CNF Arrays on PCU

To construct micro-patterns of CNFs on PCU, a novel imprinting method was used[8]. For CNF alignment, PCU was melted by chloroform, and was coated on a glass surface. After chloroform evaporation, a Au grid (with 22 μm width spacings) was attached. Dispersed CNFs in ethanol were then placed into the spacings of the grid, the Au grid was removed from the PCU surface, and patterned CNF arrays on PCU were subsequently made.

Surface Characterization

Images of micro-aligned CNF patterns on PCU were evaluated by fluorescence microscopy (DM IRB, Leica) and on a FEI NOVA nanoSEM field emission scanning electron microscope.

Cell Culture

Substrates were sterilized in an autoclave and were exposed to UV light for 24 hours before cell culture. Osteoblasts (CRL-11372, American Type Culture Collection, population numbers 2-5) were cultured on the different substrates under standard cell culture conditions (i.e., a 37 °C, humidified, 5% CO_2/95% air environment). Osteoblasts were cultured in Dulbecco's modified eagle medium (DMEM, Gibco), supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin/streptomycin (P/S, Hyclone) under standard cell culture conditions. Human osteoblasts were seeded at a density of 2,000 cells/cm^2 (sub-confluent) onto each substrate and were incubated under standard cell culture conditions in osteoblast growth media (DMEM, 10% FBS, and 1% P/S) for 2 days. At the end of the time period, non adherent cells were removed by rinsing in PBS while adherent cells were fixed with 4% formaldehyde (Fisher) and were stained with Rhodamine Phalloidin (R415, Molecular Probes) to visualize F-actin filaments and Hoechst dye (33258, Sigma) to visualize the nucleus. Cell images were taken using fluorescence microscopy (DM IRB, Leica) with two different excitation wavelengths (400nm and 550nm) to visualize the cell nucleus and f-actin filaments, respectively. Experiments were conducted in triplicate and repeated at least three times.

Osteoblast Calcium Phosphate Mineral Deposition

To determine calcium phosphate mineral deposition on the micro-aligned CNFs in PCU substrates, osteoblasts were cultured (seeding density: 600,000 cells/cm^2) in DMEM supplemented with 10% FBS, 1%P/S, 10mM β-glycerophosphate (Sigma), and 50μg/ml L-Ascorbic Acid (Sigma) under standard cell conditions for 21 days. Osteoblast media was replaced every other day. After that time period, cells were lysed with three freeze-thaw cycles in deionized water to leave only the calcium phosphate crystals deposited by osteoblasts. EDX (Energy Dispersive X-ray) analysis was completed using an Oxford INCA 250 detector and software (Oxford Instrument America, Inc., Concord, MA).

RESULTS

Aligned CNF in PCU

As expected, fluorescence microscopy results of this study showed highly aligned micro-arrays (20µm) of CNFs successfully patterned onto PCU [Figure 1, a].

Selective Adhesion of Osteoblasts on Micro-patterns of CNFs on PCU

The results of the present study provided evidence of selective osteoblast adhesion and alignment on CNFs compared to PCU [Figure 1, b and c]. Specifically, more than 80% of the osteoblasts adhered on CNF arrays but less than 20% on the PCU portion of the surface.

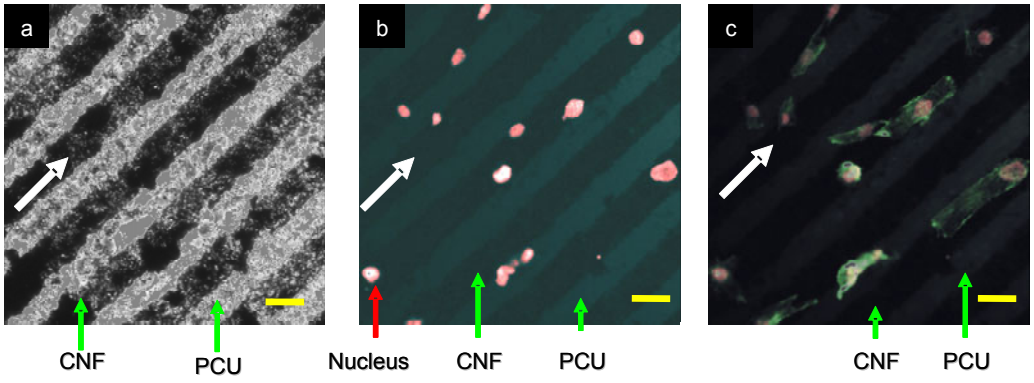

Figure 1-a) Prior to osteoblast adhesion on aligned CNF on PCU. b) Selective adhesion of osteoblasts on CNF on PCU. c) Aligned osteoblast adhesion (all bars are 30 µm). All arrows show aligned direction of CNF arrays.

Selective Deposition of Calcium Phosphate Minerals by Osteoblasts on Micro-patterns of CNFs on PCU

After 21 days of osteoblast culture, directed deposition of calcium phosphate minerals was observed on CNF compared to PCU micro-patterns using SEM and EDS [Figure 2]. Thus, the results of this study demonstrate the ability to mimic the alignment of hydroxyapatite in bone on micro-patterned CNFs on PCU. Such alignment resulted in directed osteoblast adhesion and subsequent calcium phosphate mineral deposition on

CNF regions. Moreover, since this occurred on the conductive region of the substrate (CNFs), it is possible that future studies could use applied voltages to further improve osteoblast function. Lastly, since adhesion is a prerequisite for osteoblasts to deposit calcium, it was expected that the preferred attachment of osteoblasts on CNF over PCU regions would translate into preferred calcium phosphate mineral deposition directed on CNF micro-patterns.

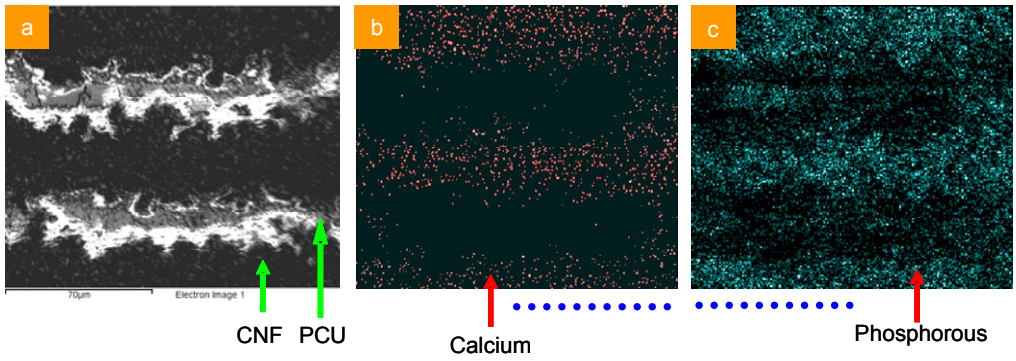

Figure 2-a) SEM image of aligned patterns of carbon nanofibers on PCU after 21 days of cell culture. b) EDX mapping of aligned calcium patterns on PCU after 21 days (Red dots show strong calcium signals on CNF compared to PCU region. c) EDX mapping of aligned phosphorous patterns on PCU after 21 days (Blue dots show strong phosphorous signals on CNF compared to PCU region, blue bars are 70µm).

CONCLUSIONS

In conclusion, we observed selective osteoblast adhesion on aligned patterns of carbon nanofibers (CNFs) on a polymer (PCU) matrix. Moreover, we observed enhanced calcium phosphate mineral deposition by osteoblasts along CNF micro-patterns on PCU matrices. These results demonstrated the optimal interactions osteoblasts have with CNFs. Their ability to increase osteoblast function may be used as novel implant nanophase materials in bone tissue engineering. Lastly, these results strongly suggest that CNF micro-patterns in PCU should be further studied for orthopedic applications in order to mimic the anisotropic alignment of hydroxyapatite in long bones.

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