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A Novel Approach to Control Growth, Orientation, and Shape of Human Osteoblasts

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A NOVEL APPROACH TO CONTROL GROWTH, ORIENTATION AND SHAPE OF HUMAN OSTEOBLASTS

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Abstract

Carbon-based materials are considered to be promising materials as implants because of their unique mechanical and biocompatibility properties. The current paper investigates the use of carbon-based materials as a functional interface for tissue scaffolds and medical implants. Three basic parameters were explored such as graphene orientation, crystallinity and surface interaction. To explore the effect of the orientation, samples were made with and without a preferred carbon orientation. Conversely, the crystallinity was studied using graphitic and carbonaceous matrices. Fluorescent, confocal and environmental scanning microscopy was used to visualize cell response. The cell attachment, proliferation and elongation were prevalent on the unidirectional carbon preform. It seems that the cells tended to orient parallel to the fiber axis (i.e. parallel to the 002 graphene plane) and proliferate as a function of higher crystallinity. In conclusion, the osteoblast (the bone-forming cells) attachment and growth rate is a function of carbon structure, more specifically, the crystallite size, graphene orientation and carbon graphitizability.

Introduction

In the growing need for superior implants, prosthetics and scaffolds, biomaterials have become a highly explored field. These artificial materials are an integral part in this research effort, allowing for the study of cell attachment, growth, differentiation, functioning, viability, and matrix degradation.[3,30,32,33,44,45] Biomaterials must fulfill 3 criterion: (i) biofunctionality-suitable physical and chemical properties for replacing the tissue, (ii) biocompatibility-no negative interactions from the materials to the biological environment, and (iii) inert to the biological environment or degradable by the system.[41]

Past studies have addressed the issue of carbon-based materials and their biocompatibility *in vitro* and *in vivo.*[10,35,36,37] However, many groups have explored carbon materials as a black box; materials that allow for cell attachment and growth but without any incite to the inner workings of the material and its structure. There has been very limited amount of research done to explain the carbon material; its structure and orientation, related to preferred cell attachment, growth and proliferation. Several studies relate surface roughness to the increase in attachment of osteoblasts.[5,11,16,29,37] Compared to titanium and cobalt alloys, nanometer dimension carbon fibers promoted osteoblast attachment.[37] Furthermore, the same study also reported that smooth muscle cell, fibroblast, and chondrocyte attachment decreased with an increase in either carbon nanofiber surface energy or simultaneous change in carbon nanofiber chemistry.[37] Using the surface energy as an inhibitor for competing cells could provide a more ideal scaffold for bone regeneration.[37]

In recent years the use of carbon-based materials in medical applications has become more prevalent. Carbon materials have been established as the most advantageous substrates for attachment and growth of several types of cells in vitro as compared to titanium and cobalt alloys [26,37], Teflon and Dacron fabric [40] and polymers.[5,36] Carbon fibers and graphitic material are two commonly used carbon-based materials. Even though both are chemically inert and biocompatible there are many differences between the graphite structure and carbon fiber, which could influence their environmental interaction. Structure, for example, controls the properties of the final product and in carbon fibers this structure could be controlled and modified to produce fibers with different material properties.[17] However, one very important structural difference is that, at an atomic level, the degree of structural ordering in the carbon fibers is present only in the basal planes whereas in graphite it is present in all directions.[18,22,34] Furthermore, in graphite, bonds within the sheets are very strong, but interactions between the sheets are weaker and can be broken easily, leading to the brittle nature of graphite composites and the lack of structural integrity.[9,18,22] Carbon fibers, produced by textile technology, have been considered for hard: dental [24], facial defect [31], internal fixation [42] and soft: ligaments [8], tendons [46], cartilage [27] and endosseous dental [2], tissue implants. Studies using carbon fibrous implants suggest that carbon fibers do not inhibit cell growth, and can act as a scaffold for tissue proliferation.[8,20] In addition, previous *in vivo* studies have

shown that carbon fibers produced through the use of polyacrylonitrile (PAN) precursors are promising for use within the body [10,31], specifically for orthopedic applications.[10] Results provided evidence that material characteristics such as a less crystallized single carbon fiber as well as a more basic chemical composition contributed to improved compatibility and regeneration of both soft (i.e., ligaments and tendons) and hard (i.e., bone) tissues.[10] Blazewicz *et al.* [10] also studied the biological response that carbon fibers, which varied in processing temperature, had when implanted into subcutaneous tissue. Reports seemed to demonstrate a correlation with the degree of crystallinity, the graphite structure and the amount of assimilation in the body. However, the structural component was not addressed; the higher the processing temperature the more brittle the material becomes and the structural integrity is decreased. This could have an impact on how well the body assimilates the material. Furthermore, carbon fibers were studied as a stabilization material in keratoprosthesis and fibroblast cells exhibited strong adherence to the carbon fibers.[23] Abusafieh *et al*.[1] and Lewandowska *et al*.[28] have demonstrated that carbon fibers could be used in bone defect treatment because of the excellent mechanical properties in dry and swollen environment. Carbon fiber use in the repair of joint surfaces and as scaffolds [13,39] has also been explored and in a review by Christel *et al.* [15] carbon fibers were considered to be a good material for total hip replacement and internal fixation in the form of composites. Carbon fiber reinforced polymer composites have been synthesized that closely match the tensile strength and modulus of bone.[14] This property has the potential to contribute in the production of scaffolds and implants for bone regeneration. Carbon-carbon composites [4,6] have been advantageous *in vivo* because of their unique biocompatible, physical, and chemical properties. Furthermore, carbon fiber reinforced carbon composites (CFRC) are very promising materials because of the pure carbon base and excellent mechanical properties.[18,19,43] All these applications are based on the unique properties of carbon as compared to other biomaterials-titanium, cobalt and stainless steel. The current study attempts to find novel methods for controlling the attachment, growth and proliferation of human osteoblasts (bone-forming cells). With hopes to obtain a fundamental understanding of the material structure and orientation related to osteoblast response using two dimensional carbon materials: carbon fiber-epoxy composites, carbon-carbon composites, and graphitic carbon materials as model materials. Moreover, the use of PAN, graphitic, and functionalized PAN carbon fibers as possible tissue scaffold materials is also examined.

Materials and characterization methods

Material fabrication

Carbon fiber/epoxy composites (CFEC) were prepared with 50% carbon fiber and 50% epoxy resin that was then cured. Carbon-carbon composites (CCC) were prepared using carbon fiber performs that were densified using chemical vapor deposition method to reach a density of 1.84g/cc. Graphite material (SG) was made using a sintering technique by pressing graphite powder with mesophase pitch as a binder. The sintered piece was carbonized and graphitized to 2600^{o}C. Small 2cm x 2cm x .2cm pieces were prepared for tissue culture assays. The materials were rinsed in distilled and deionized water before use. Carbon samples were sterile and subjected to an *in vitro,* 37^{o}C culture environment. Two-dimensional sample surfaces were uncoated and chemically inert to provide consistency throughout the study. The carbon-based samples were not subjected to any loading during the study to minimize the mechanical effect on the structural integrity.

Cell Culture

Primary human osteoblasts (ATCC) were maintained in culture with Dulbeccos's modified Eagle's medium (Sigma Aldrich, St Louis MO), DMEM, supplemented with 10% (v/v) fetal calf serum, 0.02M of HEPES buffer, 2mM of glutamine, 0.5mM sodium pyruvate (Atlanta Biologicals, Atlanta GA), 2.5mM L-glutamine (Invitrogen, CA) and G418 Sulfate Antibiotic 0.3mg/ml (Fisher Scientific, NJ). Samples were rinsed in sterile phosphate buffered saline (Hyclone, UT), PBS, prior to incubation with cells. Small, 4cm2 pieces were place in sterile 35mm cell culture dishes (Corning, New York). Confluent flasks of osteoblasts were incubated with trypsin/HEPES (0.02% trypsin, 10mM of HEPES in Ca, Mg-free PBS) for 5 minutes, spun down at 1000 RPM for 5 minutes and then resuspended in fresh culture medium. Osteoblasts were seeded onto surfaces at a density of 50,000/cm2. These samples were incubated in culture medium at 37^{o}C, 5% CO2.

Cell Attachment and Proliferation Assays

Cell attachment and proliferation was measured using fluorescent microscopy. Cells were seeded at a density of 50,000/cm2 and incubated on carbon-based material surfaces at selected time points: 2h, 24h, 48h, and 96h. Cell attachment was calculated at the 2-hour time point. Following the incubation the media was removed and

the samples were rinsed with PBS to remove any non-adherent cells. Samples were fixed with 3% formaldehyde for 15 minutes, rinsed with PBS, followed by a 15-minute permealization step with cold methanol. Samples were rinsed again in PBS and stained with Hoechst Stain (Invitrogen, CA), 10ug/ml, for 45 min. Samples were coated with Prolong Gold (Invitrogen, CA) to prevent loss of fluorescence. The samples were excited at 350nm wavelength and read at 450nm using a Nikon fluorescent microscope. Random areas of 1.13×10^6 μm^2 for the CEFC, CCC and SG samples were counted. The results of the 2 experiments (3 samples/material/experiment) were pooled and averaged. Total cell count was calculated using the sample area.

Microscopy characterization

Samples seeded at a density of 50,000/cm2 were grown for 4 days and morphological characterization was carried out using an environmental scanning microscope (ESEM). Structural characterization was carried out using a confocal microscope. Two-dimensional carbon-based samples and carbon fibers alone were seeded with cells and incubated for 2 and 8 days respectively at 37^oC, 5% CO_2. Following this step the samples were washed with PBS to remove any non-adhered cells and fixed in 3% formaldehyde for 15 min followed by a 15 min permealization step in cold methanol. The cells were washed again in PBS to remove excess methanol and stained with Alexa Fluor 488 (Invitrogen, CA), a filamentous actin stain; green in color, for 30 min. The samples were then washed with PBS and stained with TO PRO-3 (Invitrogen, CA), a nuclear stain; blue in color, for 30 min. Finally, the samples were rinsed with PBS and coated with Prolong Gold (Invitrogen, CA) to preserve fluorescence. Samples were visualized and photographed using an Olympus confocal microscope with excitation wavelengths of 488 nm and 642 nm and emission wavelengths of 525 nm and 661 nm for Alexa Fluor 488 and TO PRO-3, respectively.

Statistical Analysis

The results were expressed as means ± SD from two pooled experiments of 3 samples for each experimental group. Multiple comparison procedures were made by one-way analysis of variance (ANOVA). The p values equal to or less than 0.05 were considered significant.

Results/Discussion

Increased osteoblast attachment and proliferation could lead to better integration of the implant/scaffold *in vivo*. Osteoblast attachment on carbon-based surfaces after a 2-hour incubation was examined by fluorescence microscopy. The three different carbon-based materials: carbon fiber/epoxy composite (CFEC), carbon-carbon composite (CCC) and sintered graphite (SG), exhibited different cell responses across the surface of the material. Figure 1 shows the cell density that attached on each of the sample surfaces. It is apparent that the cell attachment is more developed and prevalent on the CFCC and CCC samples than the SG sample. Furthermore, it appears that even at 24 hours attachment is the predominant response. At 48 hours there is a significant difference in cell density and proliferation increases dramatically (Figure 2). As time increases the cell density also increases (Figure 2). The carbon content in each of the three materials is represented in Table 1. The area of carbon-species available for attachment on the CFEC is significantly less then on the other materials. Based on this condition the CFEC exhibited the highest cell attraction on the surface of the material (Table 1, Figure 2). Carbon surfaces have been shown to exhibit higher osteoblast densities in attachment and proliferation as compared to titanium and cobalt alloys.[37] Several studies reported that the surface properties play a critical role in cell attachment and proliferation.[3,4,11,16] Specifically, one group reported that the higher surface free energy on carbon composites increased the cell density.[35] Another group studied the attachment of osteoblasts with respect to surface roughness of carbon nanofiber compacts and found an increase in attachment of osteoblast cells to materials with increased nanometer surface roughness.[37] Several other studies have also reported that surface roughness is an integral factor in the determining the degree of attachment and proliferation of osteoblast cells.[4,11,16,29] In other studies, however, surface roughness was reported to have no significant effect on osteoblast attachment, growth, and proliferation.[4,25,29] Furthermore, some groups have even reported that the attachment and proliferation of other cell types-fibroblasts, smooth muscle, and chondrocytes is affected by surface roughness [37] while others have reported that there is no significant connection between surface roughness and attachment or proliferation.[38] Agreement with experiments has been variable. This suggests that cellular interaction could vary between material surfaces and cell response might not be accurately measured using only this parameter. Moreover, surface properties could contribute to the attachment, proliferation and inhibition of cells; nevertheless, there is no clear understanding of why the results from these studies tend to differ. More importantly, surface roughness is not the only factor involved in the control of attachment and proliferation and other areas need to be explored. There seems

to be other material properties involved, primarily, the degree of crystallinity, the grain orientation, and the fiber orientation, all of which are explored in the current study.

Table 1 describes the crystallinity and the fiber orientation of the carbon materials. The influence of the degree of crystallinity and fiber orientation or grain orientation is observed in Figure's 1 and 2. As the degree of crystallinity increases the number of adhered cells decreases. CFEC has the lowest degree of crystallinity followed by CCC and SG. Moreover, from table 1, the fiber orientation of the materials is different. SG does not have a fiber-oriented structure while CCC and CFEC have multidirectional and unidirectional structures, respectively. Therefore, it could be suggested that the unidirectional orientation is the preferred orientation for cell attachment.

Osteoblast proliferation on carbon-based samples was measured for 4 days. There was a significant difference in proliferation between the three materials. Figure 2 illustrates the proliferation of the cells between the different materials in the 4-day study. In the case of CFCC and CCC we could observe that there is a network or a directional growth that could be related to the carbon fiber architecture and orientation (Figure 1). Bacakova *et al.* [4] noted that osteoblasts grown on carbon fiber-reinforced carbon composites aligned parallel to the carbon fibers. Similar results were obtained in our study but moreover we noticed a directional growth along the axis of the fiber. There might be preferential active sites that enhance or inhibit the cell growth. Using high magnification imaging we observed that cells grow ultimately on the carbon material and preferentially on the carbon fiber (Figure 3). They definitely do not grow on the epoxy matrix (Figure 3).

Changing the fiber orientation of the material influences the degree of proliferation of osteoblasts. Table 1 points out that CFEC has half as much carbon content as the other two materials nevertheless has the highest proliferation (Figure 2). As can be seen in Figure 3, an ESEM image of osteoblasts grown on carbon fiber, the cells are attaching and growing on the fiber axis and not on the epoxy matrix. This proliferation of cells on carbon alone needs to be stressed. With this 50% decrease in area (Table 1) on which the cells could attach and grow and the observation that CFEC had the highest cell proliferation we can suggest that the unidirectional carbon fiber orientation increases cell growth. The grains of the carbon fiber are positioned in such a way to provide increased attachment and growth. The C/C composite on the other hand had a similar cell count at the various time points but the carbon content was increased by 100 percent from the CFEC. This less oriented structure decreased the amount of cell growth per area. For the sintered graphite material, there is no preferred orientation of the graphite grains into a given axis, which leads to a decrease in growth and proliferation. The strong influence of the preferred orientation could have an impact on the structural design of scaffolds and implants.

Furthermore, there was also a variation in cell structure between the three materials. Cells on the CFEC grew along the fiber axis and were mainly elongated and spindle shaped (Figure 4A). Bacakova *et al.* [4] reported similar results; they do tend to be spindle shaped and that the cells attach on a small contact area. However, for the CCC and SG samples, the cells grew in a more round or polygonal shape (Figure 4B, C). Since the materials did not have any functional groups attached at the surface we are suggesting that the carbon itself is the key component. The surface seems to have active sites available for binding and this leads to the preferential binding of the cells to the carbon. The active sites will determine the direction of growth on the surface of the material. However, attachment and proliferation of cells will decrease if few active sites are available on the surface of the material.

Conclusion

In conclusion, there have been many studies performed with relation to carbon materials and osteoblast attachment, growth, and proliferation.[3,30,32,33,35-37,44,45] All of these studies have reported that osteoblasts adhere to carbon surfaces and proliferate but the density varies between materials. The novelty in the current study is that osteoblasts not only adhere to the carbon material but that the attachment, proliferation, cell shape and direction of cell growth is dependent on the crystallinity of the material, the grain orientation and the fiber orientation of the carbon-based materials. Moreover, by changing or modifying these properties we could control the cell response to the material. Our study reports increased attachment on carbon materials with lower crystallinity and higher grain and fiber orientation. Furthermore, the proliferation of cells was greater on carbon samples with a higher fiber orientation and there was a directional growth along the carbon fiber. Moreover, we observed that the structural characteristics (eg. shape) of the cells vary between materials and are also dependent on the grain orientation, crystallinity and fiber orientation of the carbon material. The CFEC had elongated cells whereas the other two materials, CCC and SG, had cells that were spherical in nature. The carbon surface, specifically, the active sites, have a direct impact on cell attachment and growth as well as cell structure. In addition, the cells did not grow on the epoxy resin but more importantly the epoxy resin did not contribute to the directional growth along the carbon fiber axis. The carbon material alone was responsible for this phenomenon. These results strongly suggest that there is a preferential capacity for cell attachment and proliferation and cell shape

is affected by changing material properties and needs to be further explored for use as scaffolds and implants. Also, carbon-based materials could be tailored in order to obtain optimal structures, properties and cell-interface interactions and should also be explored more in depth.

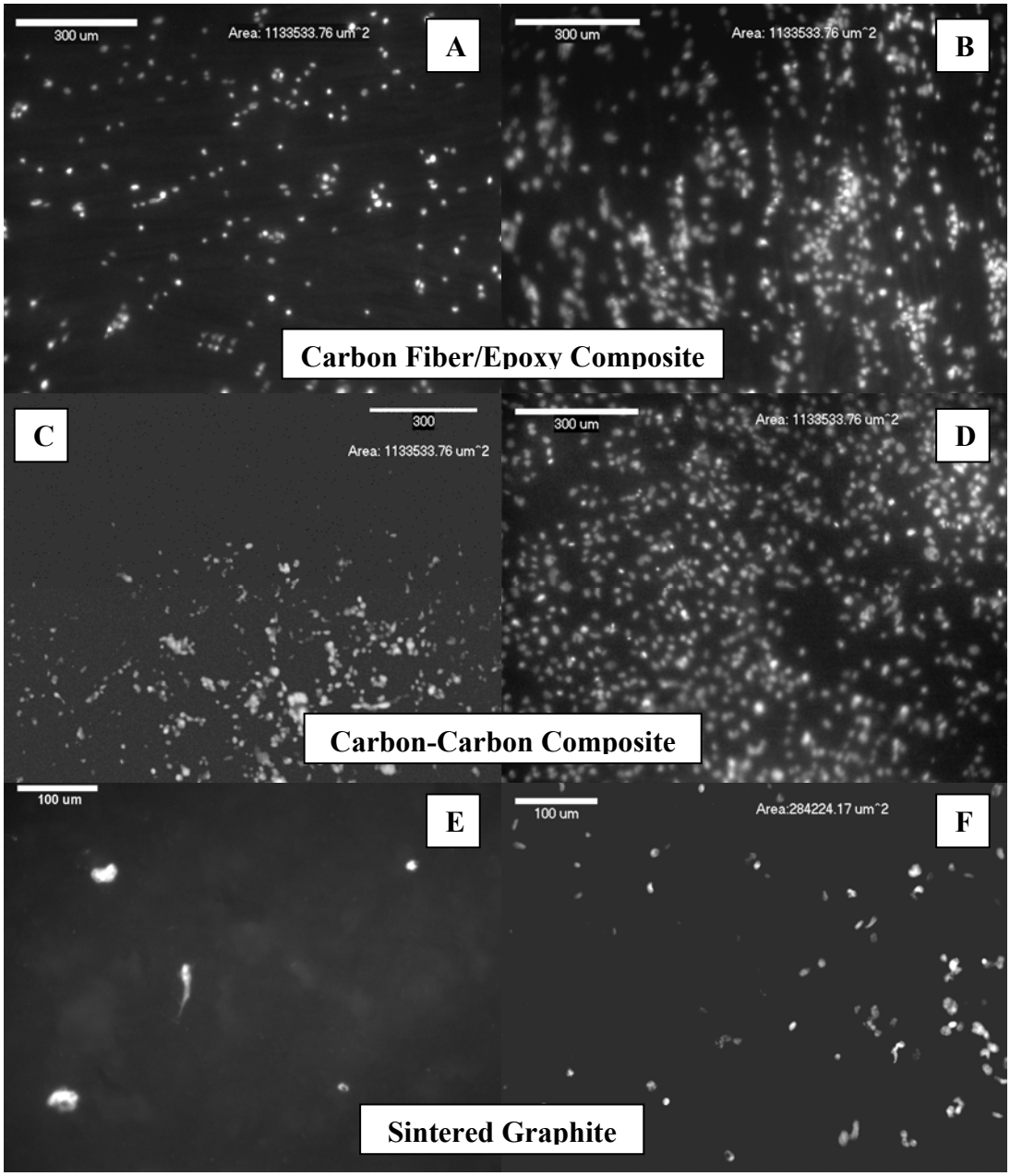

Figure 1. A and B: osteoblast growth on CFEC at 2 and 96 hours, respectively; C and D: osteoblast growth on C/C composite at 2 and 96 hours, respectively; E and F: osteoblast growth on SG at 2 and 96 hours, respectively.

Table 1. Percent difference in carbon content between the carbon-based materials and their relative orientation and crystallinity.

Material	Carbon Content (%)/cm^2	Degree of Crystallinity (1=most, 3=least)	Fiber Orientation
Carbon/Carbon Composite (CCC)	100	2	Multi-directional
Carbon fiber/epoxy Composite (CFEC)	50	3	Unidirectional
Sintered Graphite (SG)	100	1	No orientation

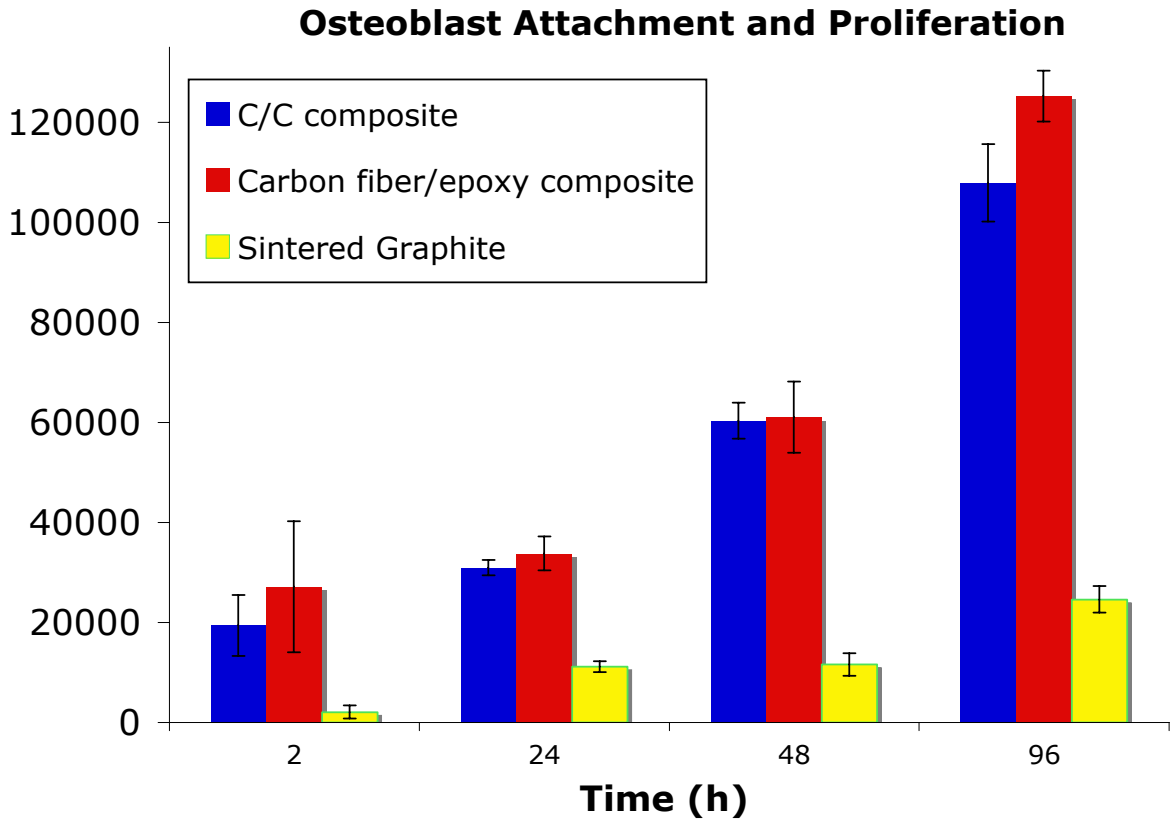

Figure 2. Attachment and Proliferation assay results: Attachment of osteoblasts at 2 hours as well as the proliferation of osteoblasts on the three different carbon-based materials in the 4-day study. Based on carbon content/area the attachment and rate of growth of osteoblasts on CFEC should be doubled because it has half as much carbon per area as compared to CCC and SG. CFEC has the highest attachment and proliferation. Data represents the mean ± SD of pooled data from 3 experiments; n = 3/experiment. CC composites and CFEC composites significantly different ($p \leq 0.05$) than SG.

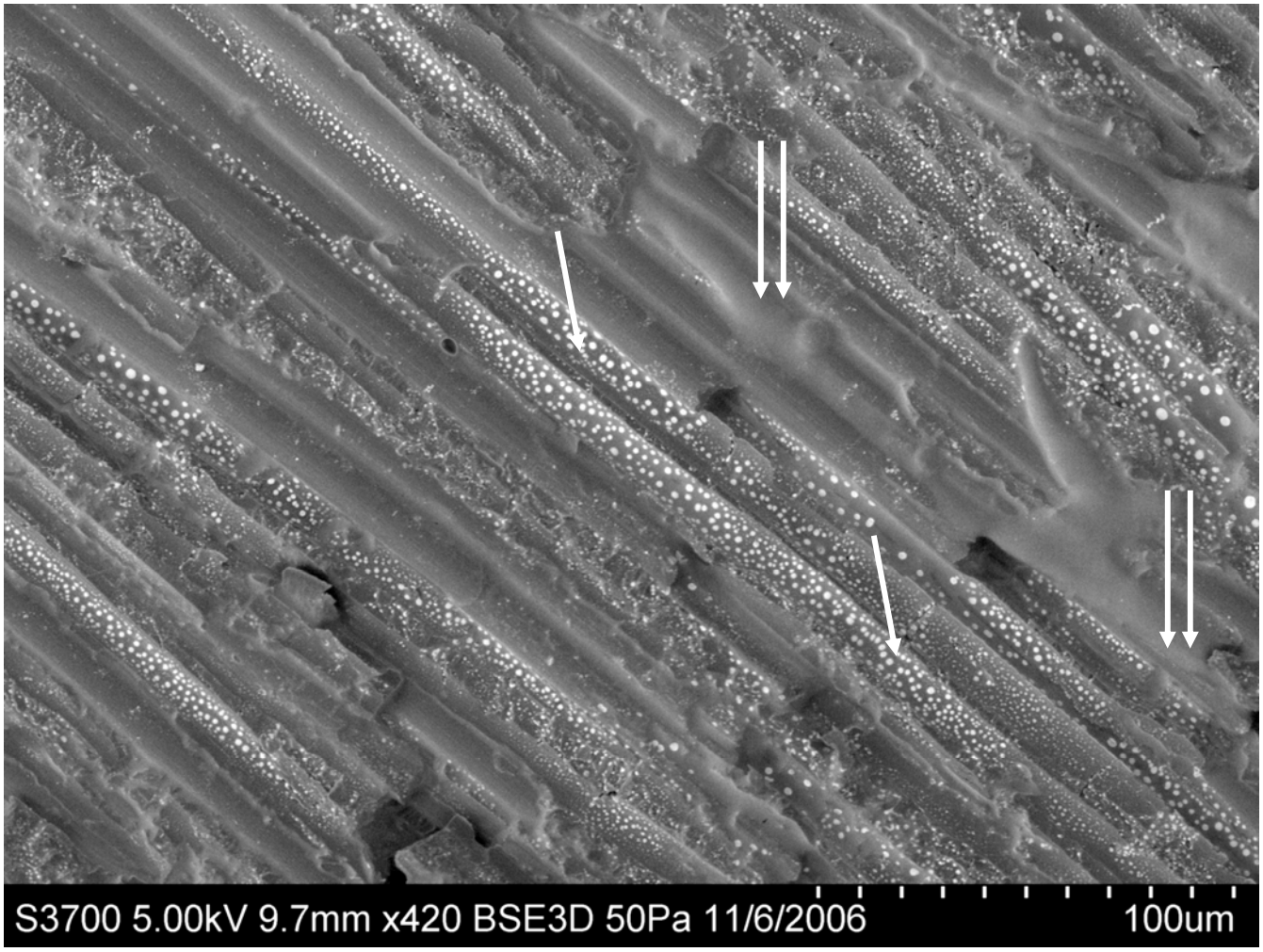

Figure 3. ESEM image of carbon fiber/epoxy composite with osteoblast cell growth. Single arrow: carbon fiber with increased cellular growth as can be seen with the white dots. Double arrows: epoxy matrix with no cellular growth.

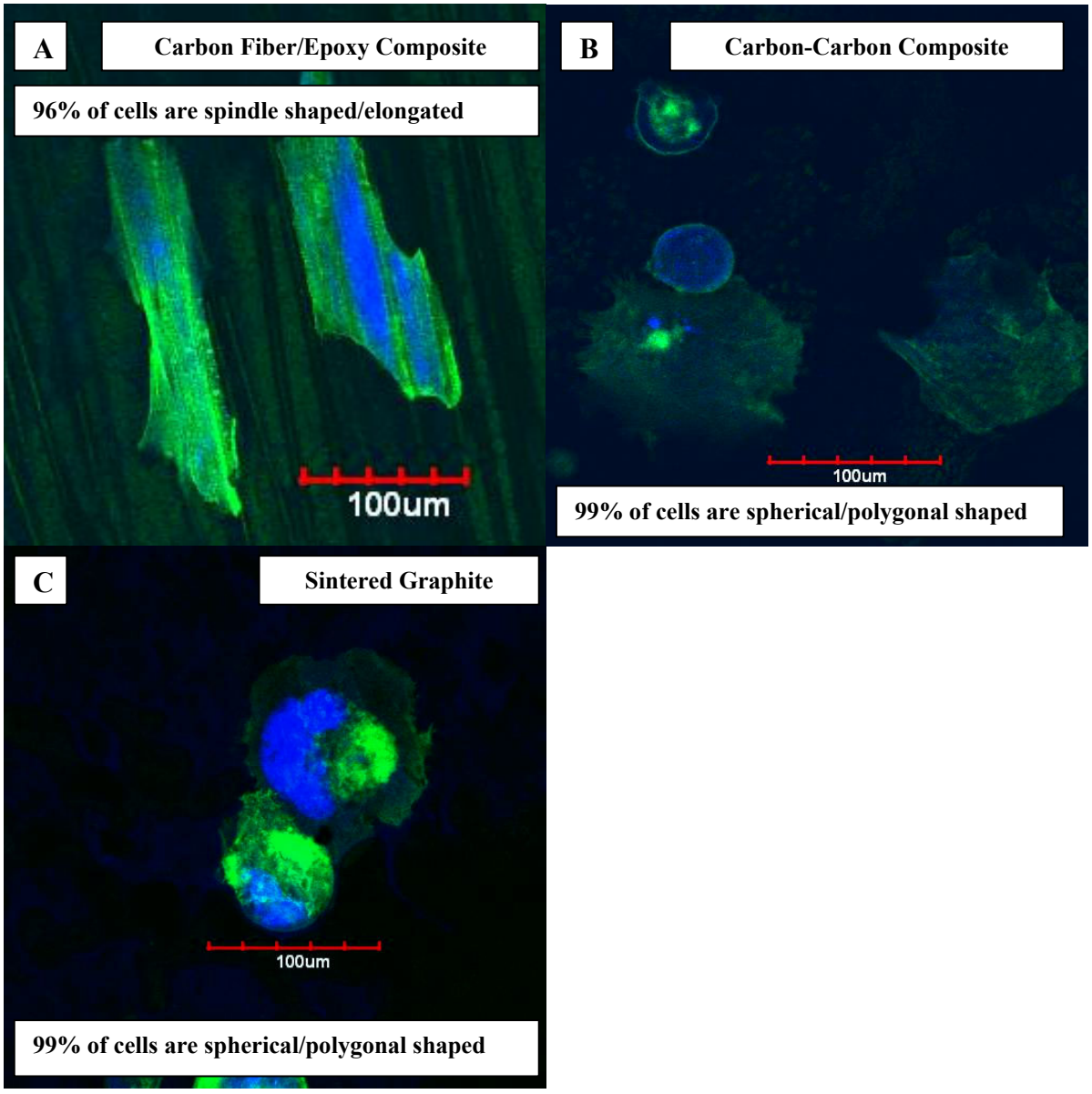

Figure 4. Structural characterization at 48 hours; A: osteoblasts grown on CFEC (elongated shape), B: osteoblasts grown on CCC (spherical or polygonal shape), C: osteoblasts grown on SG (spherical or polygonal shape). Staining: green color represents f-actin (Alexa Fluor 488 stain), blue color represents the nucleus (TO-PRO 3 stain).

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