Developing Biosensors for Monitoring Orthopedic Tissue Growth

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Abstract

The objective of this present study was to create a biosensor which can monitor in situ orthopedic tissue growth juxtaposed to a newly implanted orthopedic material. This biosensor has unique properties including the ability to sense, detect, and control bone regrowth. Such a biosensor is useful to not only regenerate tissue necessary for orthopedic implant success but it also aids in informing an orthopedic surgeon if sufficient new bone growth occurred. If the sensor determines that insufficient new bone growth occurred, the sensor can also act in an intelligent manner to release bone growth factors to increase bone formation. The primary biomaterial in this biosensor is anodized titanium, developed by chemical etching, and passivation treatments. Carbon nanotubes (CNTs), in terms of their electrical and mechanical properties, are imperative considerations when designing such biosensors since they will be used to apply and measure conductivity changes as new bone grows next to the implant. For this, parallel multiwall CNTs were grown from the pores of the anodized titanium by the chemical vapor deposition process. Lastly, this sensor is composed of a conductive, biodegradable, polymer layer that degrades when bone grows and, consequently, undergoes a change in conductivity that can be measured by the CNTs grown out of the anodized titanium. This conductive, biodegradable polymer consists of polypyrrole (which is conductive) and poly-lactic-co-glycolic acid (which is biodegradable). Preliminary *in vitro* results suggest that osteoblast functions (adhesion and proliferation) on such a biosensor is not significantly compromised when compared to currently-used titanium, yet, retains the ability to potentially measure new bone growth juxtaposed to an implant. In addition, although not tested here, it is anticipated that bone growth could be enhanced on these biosensors electrically.

Introduction

In the human body, bone matrices are generally 90% wt. fibrillar type-I collagen, and 10% wt. hydroxyapatite crystals. Bone usually consists of living cells: osteoblasts, osteocytes, and osteoclasts, which are located in bone's nanostructured mineralized organic matrix [1]. Osteoblasts form the organic matrix of bone, and produce alkaline phosphate, which plays a critical role in the mineralization of bone. When they are trapped in the bone, which they formed, osteoblasts differentiate into osteocytes. While osteoblasts make bone, osteoclasts break it down, releasing acid that decomposes calcium phosphate-based apatite minerals [2]. Undoubtedly, implants require the functions of osteoblasts to create new bone growth in situ. However, in orthopedics, many kinds of materials are used (such as CoCrMo, Ti6A14V, and Ti) each of which result in varying forms of new bone growth.

Titanium (Ti) is well-known for its high strength-to-weight ratio, and consequently is used in orthopedic applications and maxillofacial surgeries. Not only are the mechanical properties of Ti (such as stiffness, high load resistance, fatigue resistance and ductility) sufficient, but its biocompatibility properties make it an attractive material for orthopedic applications. Critical in the design of successful biomaterials is the ability of such materials to control protein absorption, and consequently osteoblast adhesion, after these biomaterials are

implanted. The degree to which proteins absorb on implant surfaces depends on biomaterial properties, such as their chemistry, charge, wettability, and topography.

In the case of surface chemistry, oxidized layers of titanium oxide (TiO_2) are formed on top of Ti simply through its exposure to air and/or water. After implantation, oxidized Ti surfaces bind with structural water, forming $–O^-$, $–OH^-$, and $–OH_2^+$ sites, and also possess a weak negative charge at physiological pH. Therefore, this oxidized layer provides a kinetic barrier that prevents Ti from corroding, and provides a graft that allows calcium phosphate crystals, cells, proteins, and collagen to bond. However, resulting changes in topography from oxidation can be modified in order to increase surface roughness for better protein adsorption, osteoblast attachment, and osseointegration. As recent research shows, nanometer surfaces of anodized Ti can be created to enhance osteoblast adhesion [3]. In that study, Ti was anodized to create nanotube-like pores on the surface, which has higher surface energy and improved wettability as measured by contact angles between unanodized and anodized Ti [4].

The objective of our study was to build off of the success of anodized Ti for orthopedic applications to create a biosensor which can monitor in situ orthopedic tissue growth juxtaposed to a newly implanted orthopedic material. This biosensor has unique properties including the ability to sense, detect, and control bone regrowth. Such a biosensor is useful not only to regenerate tissue necessary for orthopedic implant success but also to inform an orthopedic surgeon if sufficient new bone growth occurred. If the sensor determines that there is insufficient new bone growth, the sensor acts in an intelligent manner to release bone growth factors to increase bone formation. To make this biosensor, we plan to include the following components: Ti, carbon nanotubes (CNTs), and a biodegradable conductive polymer coating. Because of the mechanical strength and relative inactivity of Ti with biological substances, it is the material of choice for orthopedics [5]. Due to their unique electrical, mechanical, and biological properties, CNTs are intriguing for orthopedic applications. CNTs have shown promise for bone implantation [6]. The conductive biodegradable polymer coating is necessary since as bone grows, conductivity will change, resulting in a measurable quantity that is sensed, and sends information to a clinician.

Such a biosensor can increase bone growth in a controlled manner since materials which incorporate electrical signals or fields stimulate bone cell adhesion, proliferation, and differentiation [7, 8]. Polypyrrole can generate electrical signals through electron transfer between different polymer chains. By combining polypyrrole and poly(lactic-co-glycolic acid), polymers which have controllable degradation can easily be prepared [9]. Poly(lactic-co-glycolic acid), PLGA, is biodegradable and biocompatible because it undergoes hydrolysis. PLGA degradation time is dependent on the ratio of the monomers used during synthesis. The higher the amount of glycolide units, the lower the time required for degradation.

Materials and Methods

I. Anodization Techniques

99.2% pure Ti (Alfa Aesar) was cut into 1x1 cm^2 squares and cleaned with acetone, 70% ethanol, and deionized H_2O. These samples were etched for 10 seconds to remove an oxidized layer on the surface of Ti with a solution of 1.5% wt. nitric acid and 0.5% wt. hydrofluoric acid. Cleaned Ti was used as an anode, while a high purity platinum sheet (Alfa Aesar) served as a cathode. Both were immersed in an electrolyte solution consisting of 1.5% wt. diluted hydrofluoric acid in a Teflon beaker. The surface of the etched Ti was placed next to

the platinum sheet at a distance of around 1 cm. This anodization system (see Figure 1) provided 20 volts for 10 minutes to create nanotube anodized holes on the Ti.

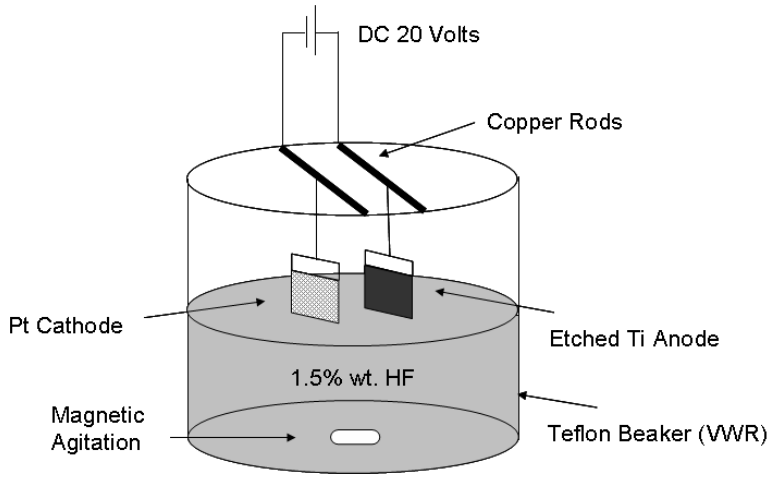

Figure 1. Schematic of the anodization process.

II. Chemical Vapor Deposition

A thermal chemical vapor deposition (CVD) (ASTEX by Applied Science & Technology Inc.) was used to grow multiwall CNTs out of the pores in anodized Ti. First, the anodized samples were dipped into 5M Cobaltous Nitrate (Allied Chemical) solution for 5 minutes, and then rinsed with distilled water and dried with compressed air. The samples were placed into a thermal CVD chamber, and then the air was pumped out for 30 minutes. The schematic CVD is shown in Figure 2. The samples were then heated up to 700 ^{o}C in a flow of 100 sccm hydrogen gas to reduce the Cobaltous Nitrate to Co for 20 minutes. After that, the H_2 gas flow was switched off and 40 sccm H_2 /160 sccm Ar was introduced into the chamber to start growing multiwall CNTs. The growth time was 30 minutes. Finally, the samples were cooled in a 100 sccm Ar flow. Initially, some of the samples were not dipped into the catalyst, Cobaltous Nitrate. However, to improve the growth of CNTs on pores of anodized Ti in terms of uniform coating and longer CNTs, the catalyst was used for other samples.

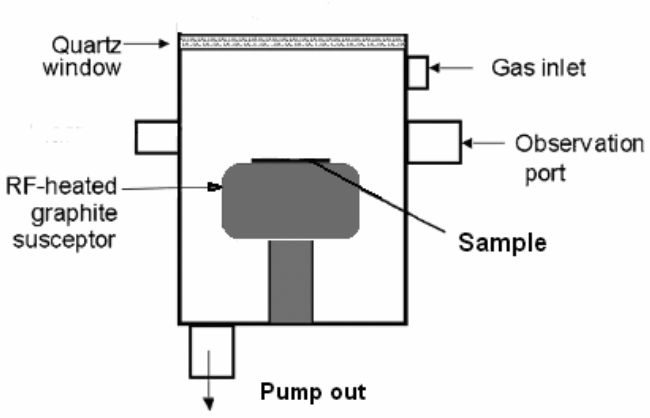

Figure 2. Schematic of the chemical vapor deposition system.

III. Cell culture

In vitro, cell biocompatibility was determined on four different kinds of samples including pure Ti, anodized Ti, CNTs grown from anodized Ti, and carbon nanopaper (buckypaper; NanoLab). Osteoblasts (CRL-11372; American Type Culture Collection) were used in the preliminary cytocompatibility tests. Cell adhesion and proliferation were determined on each substrate. First of all, the four types of samples were cleaned with phosphate buffered saline (PBS; a solution containing 8 g NaCl, 0.2 g KCl, 1.5 g Na_2PO_4, and 0.2 g KH_2PO_4 in 1000 ml DI water adjusted to a pH of 7.4; all chemicals from Sigma-Aldrich) three times, and cultured in 12 well plate, 3.83 cm^2/well, with 3,500 osteoblasts in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Hyclone), and 1% penicillin/streptomycin (P/S; Hyclone). Cells were seeded in an incubator under standard conditions (a humidified, 5% CO_2, and 95% air environment at 37° C). Osteoblast adhesion was tested for four hours in an incubator, while osteoblast proliferation was determined for one, three, and five days in an incubator. For cell proliferation tests, DMEM was changed every other day. After the specific time periods, the surrounding DMEM was removed from all samples and then the samples were rinsed with PBS three times to remove non-adhered cells. After that, all samples were rinsed with PBS three more times, and then fixed with formaldehyde (Sigma) for 10 minutes. A 4'-6-A diamidino-2-phenylindole (DAPI; Sigma) diluted solution was used to stain osteoblasts in order to observe the morphology of the cells with fluorescent microscopy using a Zeiss Axiovert 200M Light Microscope. Cell adhesion and proliferation experiments were repeated with the same procedures three times on different days.

Results

I. Material Surface Topography

As expected, after anodization, nano-sized pores were distributed uniformly throughout the Ti surfaces. The uniform pores, observed by scanning electron microscopy (SEM; Leo 1530VP) were estimated to have a diameter of around 50-60 nm and a depth of around 200 nm. Parallel multiwall CNTs successfully grew out of these anodized pores in Ti. Clearly, the topography of anodized Ti and CNTs grown from anodized Ti pores varied between in each sample. Samples with 5M Cobaltous Nitrate solution resulted in more CNTs grown from nano-pores, because this catalyst turned to deposit as Co on the bottom of each pore. Figure 3 (a) and (b) shows the topography of the samples without deposition of Co; (c) and (d) which show CNTs more uniformly in length, are the result of catalyst use. The higher density of CNTs on the Ti surfaces resulted from dipping the samples into the catalyst for extended periods of time, as well as increasing the concentrations of the catalyst solution. However, only uniform CNT grown in a proper amount is desirable, because it is more suitable for osteoblast adhesion, proliferation, and differentiation. Some regions on the surface cannot be covered by CNTs, leaving some surfaces similar with anodized Ti.

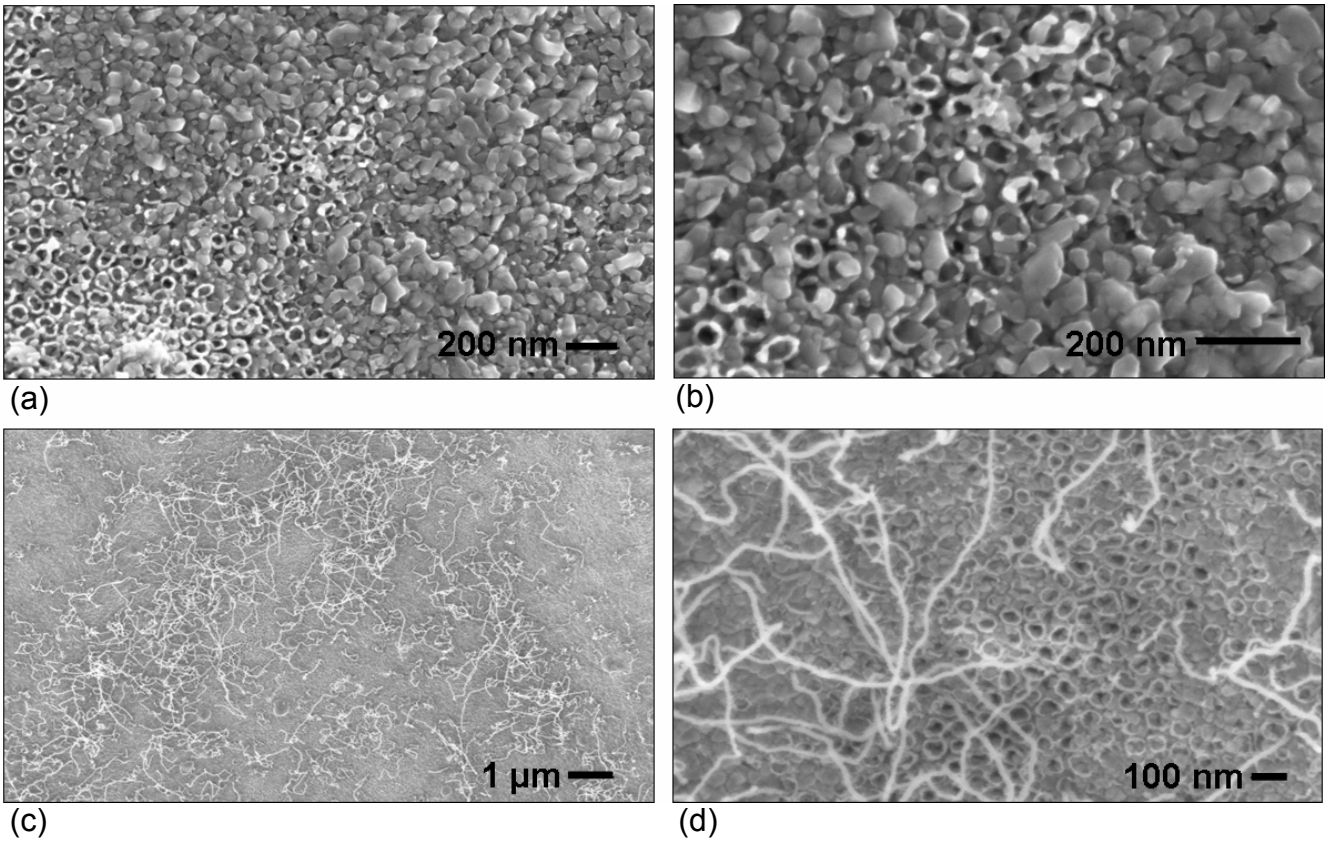

Figure 3. SEM micrographs: (a), (b) CNTs grown from the nanotubes of anodized Ti without catalyst; (c), (d) CNTs grown from the nanotubes of anodized Ti surface with catalyst.

II. Cell adhesion and proliferation

Cell adhesion and proliferation results were evaluated based on the mean value of the number of adherent cells with standard error of the mean (SE of M). The results are presented in Figure 4. Figure 5 shows fluorescent microscope micrographs of osteoblasts. The white spots are osteoblasts adherent on the different surfaces. In Figure 4, the anodized Ti samples showed increased osteoblast adhesion compared to the unanodized Ti, CNTs grown from anodized Ti, and carbon nanopaper. Osteoblast adhesion increased approximately 20% on the Ti substrates possessing nano-tube-like structures, compared to the unanodized Ti. Importantly, osteoblast adhesion on pure Ti was similar to on CNTs grown from anodized Ti. Moreover, osteoblasts proliferated and spread more on anodized Ti and CNTs grown from anodized Ti compared to pure Ti surfaces (Figure 6).

CNTs on the surfaces may decrease the ability of osteoblasts to adhere and proliferate; however, it may increase the electrical conductivity of samples which (although not tested here) would promote osteoblast function. The conductivities of Ti, anodized Ti, and CNTs grown on anodized Ti is forthcoming, as well as the synthesis of the electrically conductive biodegradable polymer coating. Osteoblast attachment on such polymers will also be determined.

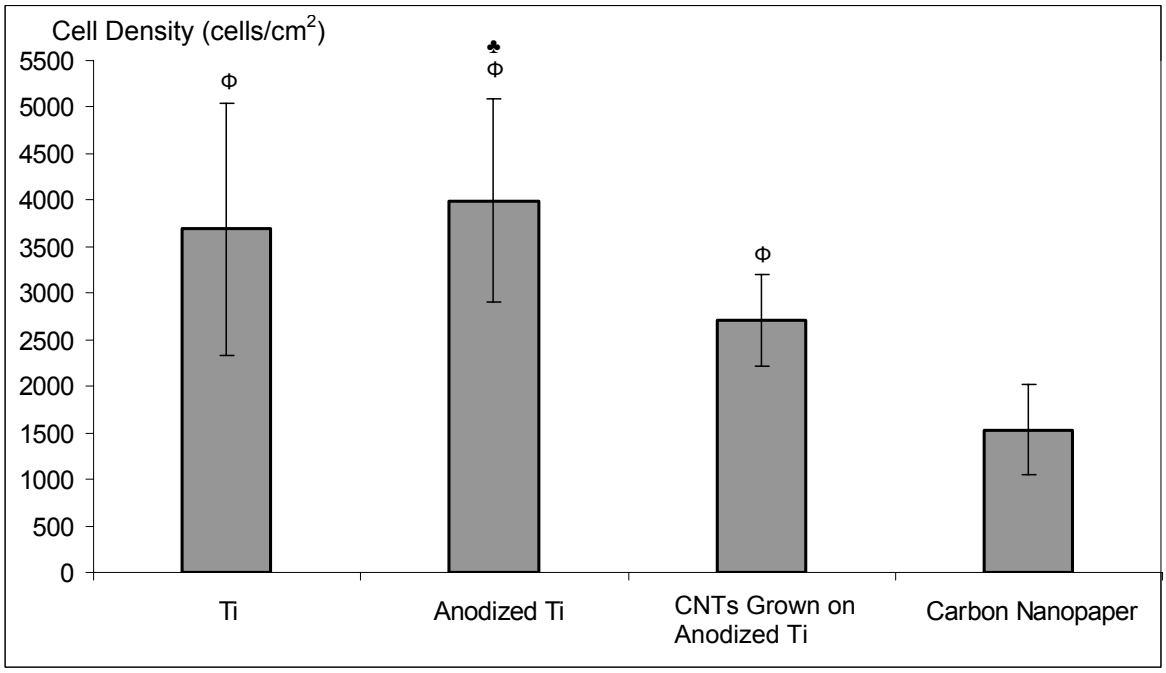

Figure 4. Osteoblast adhesion on Ti, Anodized Ti, CNTs Grown on Anodized Ti, and Carbon Nanopaper. Values are mean ±SE of M; n=3; ♣ $p<0.1$ compared to CNTs grown on anodized Ti; Φ $p<0.1$ compared to carbon nanopaper.

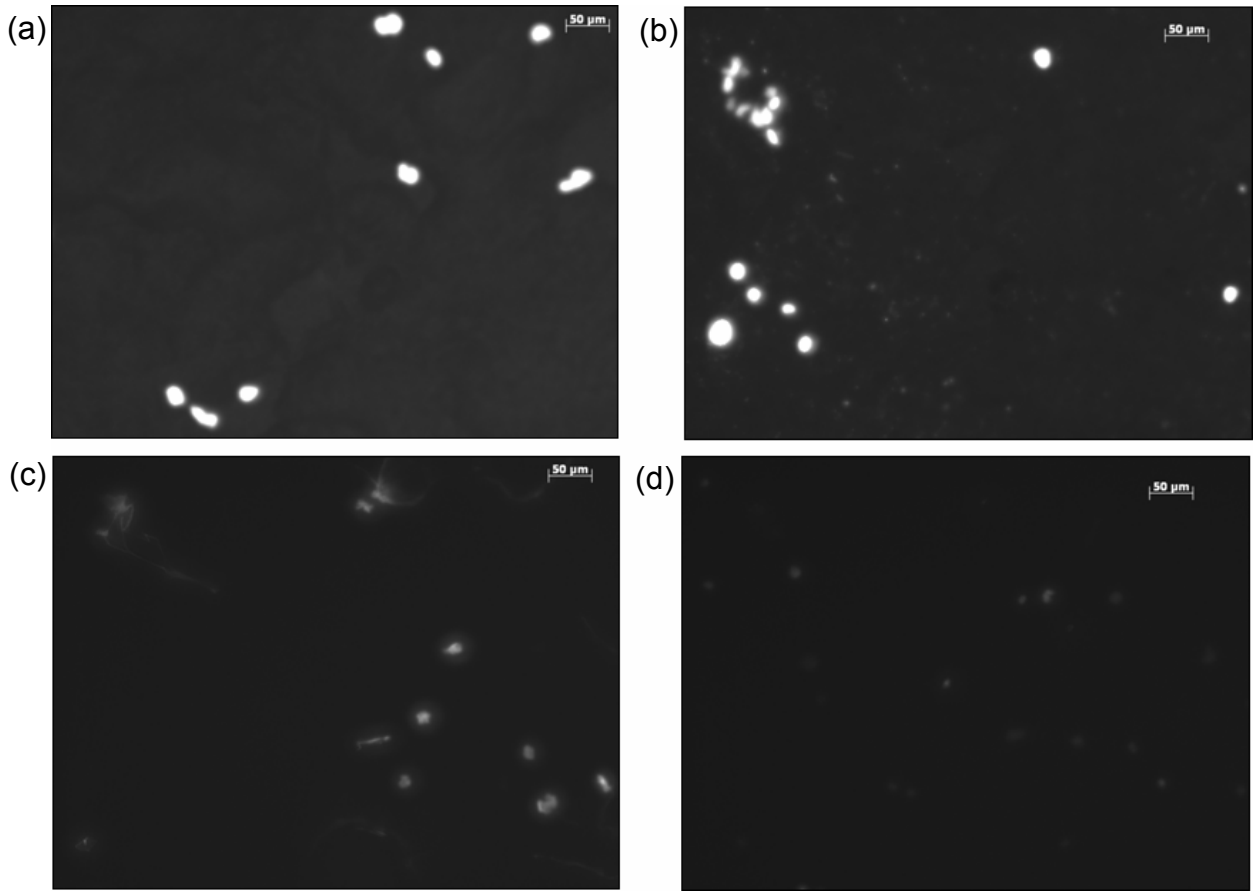

Figure 5. Fluorescent microscope micrographs of cells on: (a) Ti, (b) anodized Ti, (c) CNTs grown on anodized Ti, and (d) carbon nanopaper. Scale bars = 50µm.

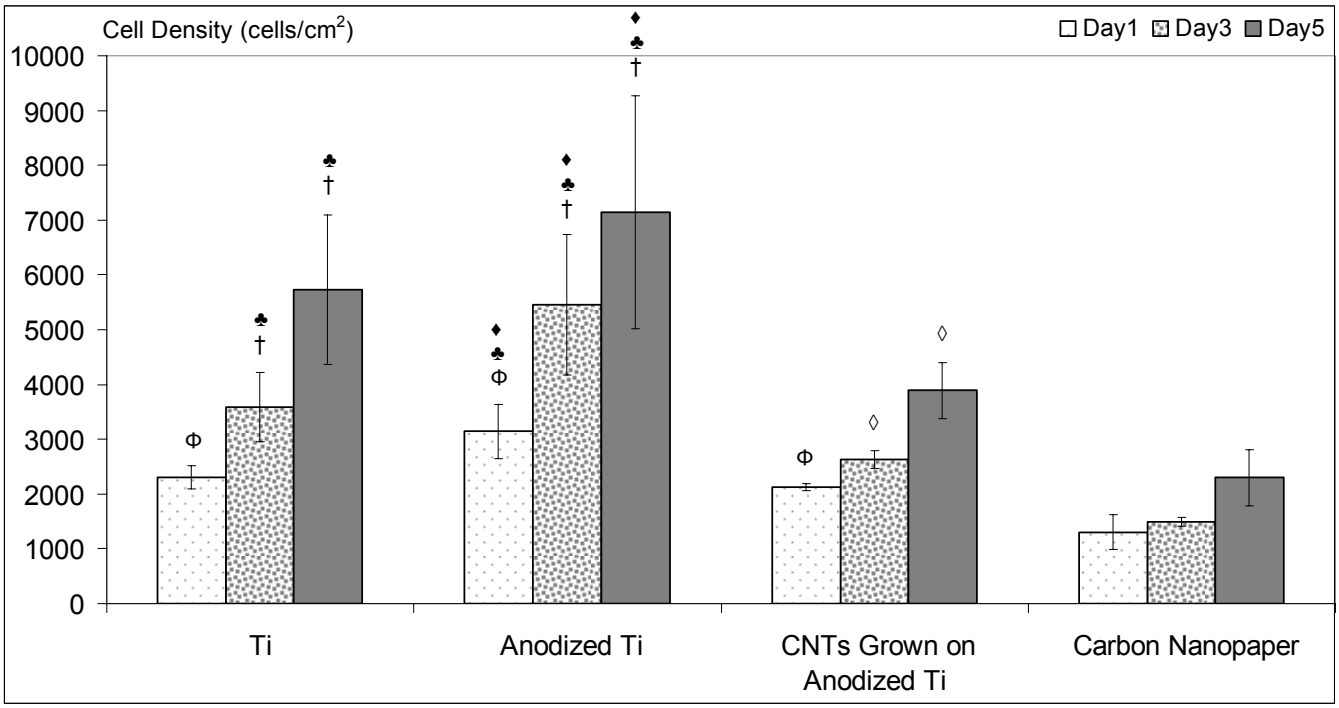

Figure 6. Osteoblasts proliferation on Ti, Anodized Ti, CNTs Grown on Anodized Ti, and Carbon Nanopaper. Values are mean ±SE of M; n=3; ♦ $p<0.1$ compared to Ti; ♣ $p<0.1$ compared to CNTs grown on anodized Ti; Φ $p<0.1$, † $p<0.05$, ◊ $p<0.02$ compared to carbon nanopaper.

Discussion

Nano-tubes on Ti were estimated about 100 to 200 nm deep, with inner diameters of approximately 70 to 80 nm when anodized under a voltage of 20 volts for 20 minutes in 0.5 % HF [10]. In our study, in which anodization occurred under 20 volts for 10 minutes, the nano-tubes were approximately 200 nm deep and 50-60 nm in diameter.

Some of the osteoblast functional differences observed between the samples of interest (shown in Figures 4, and 6) may be due to surface properties (such as changes in topography and chemistry) and/or sterilization procedures. For example, normal sterilization processes were conducted before cytocompatibility testing. Specifically, pure Ti samples and anodized Ti samples were autoclaved for 45 minutes, but CNTs grown from anodized Ti, and carbon nanopaper samples were not because of potential damage to the CNTs during heating. Instead, these samples were exposed to UV light for about 4 hours before immediately using them in cell culture testing. The number of cells on the surfaces sterilized by UV light was less than on those which were autoclaved. To compensate for that, in this study, results reported were derived from experiments in which all types of samples were sterilized by UV light only.

Recent research shows that osteoblast adhesion increased 20% on anodized Ti as compared to unanodized Ti need, led to studies to determine the reason for this increase, which measured greater vitronectin and fibronectin absorption on anodized compared to unanodized Ti [10]. Also, other studies implicate that carbon nanofibers (CNF) have good cytocompatibility properties, which promote osteoblast adhesion. Because of their high surface energy and small diameters, when CNTs are aligned on polymers, directed bone formation similar to the anisotropic natural formation of bone was observed [3, 12]. Additional study was

found that osteoblast adhesion on nanophase Ti increased due to nanometer roughness and larger percentages of particle boundaries at sample surfaces [13]. However, this study provided the first evidence of decreased osteoblast function on CNTs grown from anodized Ti as compared to plain anodized Ti.

However, although this may sound detrimental for the presently designed biosensors, this study was not conducted under electrical stimulation. On this light, CNTs have extremely desirable properties of high mechanical and thermal stability, high thermal conductivity and large current carrying capacity [14]. Researches found that bending of the nanotubes decreases their transmission function and leads to an increased electrical resistance [15]. CNTs grown on anodized Ti may increase conductivity of those samples, in which their conductivity aid in bone growth. Moreover, CNTs were prove to be important components for such nano-sensors, taking advantage of the electrical properties possessed by this carbon-based material [16]. Nanocomposites of polylactic acid and multiwall CNTs have been shown to increase osteoblast proliferation by 46% and greater than 300% calcium production when alternating current was applied to the substrate [17]. Phosphate-substituted CNTs can substitute for collagen to direct the crystallization of hydroxyapatite reaching a thickness of 3 mm after 14 days of mineralization [18]. CNTs can also be functionalized to release bioactive factors. It was shown that such factors, for example glucose oxidase, can be attached to nanotubes and still retain enzymatic activity [19]. These are promising signs for the continued use of CNT-based sensors in orthopedic applications.

Lastly, for the conductive biodegradable coating, there is a report on the synthesis and characterization of a novel polymer that possesses the unique properties of being both electrically conducting and biodegradable, thus, capable of electronically interfacing with tissues. This polymer was synthesized from conducting oligomers of pyrrole and thiophene, connected together via degradable ester linkages. The authors demonstrated that this polymer is conductive, degradable, and biocompatible [2]. We will apply such a coating to the surfaces of our CNTs grown on the anodized Ti to further develop this novel biosensor. Also, a biodegradable, electrically conductive polymer created from poly-lactic-co-glycolic acid and pyrrole will be developed and tested. Polypyrrole is not degradable, but still remains in the body long-term, and may induce chronic inflammation and require surgical removal [2]. However, another study mentioned that polypyrrole has sufficient compatibility *in vitro* and non cytotoxic properties *in vivo* for the regeneration of damaged peripheral nerves in rats [9]. Thus, the use of a conductive, biodegradable polymer coating on the presently formulated biosensors may have promised for detecting and promoting bone growth.

Conclusions

This anodization process created nano-tube pores, approximately 200 nm in depth and 50-60 nm in diameter on Ti surfaces. The preliminary results *in vitro* showed that unanodized Ti had similar osteoblast adhesion compared to anodized Ti in which CNTs were grown. For cell proliferation for days 1, 3, and 5, it was found that CNTs grown from anodized Ti samples had less cells than pure Ti, and anodized Ti. Moreover, this experiment supports an earlier study [1] that showed greater osteoblast adhesion on anodized Ti surfaces possessing nano-tube-like structures compared to the unanodized Ti. It does provide the first evidence of less osteoblast functions on CNTs grown from Ti anodized pores compared to anodized Ti alone but such results were comparable to currently used Ti.

References

1. H. M. Frost (1963), “Bone remodeling dynamics”, *Charles C Thomas Publisher*.
2. T.J. Rivers, T.W. Hudson, and C.E. Schmidt (2002), “Synthesis of a novel, biodegradable electrically conducting polymer for biomedical applications”, *Advanced Functional Materials*, pp. 33-37.
3. D. Khang, M. Sato, R.L. Price, A. E. Ribbe and T.J Webster (2006), “Selective adhesion and mineral deposition by osteoblasts on carbon nanofiber patterns”, *International Journal of Nanomedicine*, pp. 65-72.
4. X. Zhu , J. Chen , L. Scheideler , R. Reichl and J. Geis-Gerstorfer (2004), “Effects of topography and composition of titanium surface oxides on osteoblast responses”, *Biomaterials*, pp. 4087-4103.
5. G. E. Park and T.J. Webster (2005), “A review of nanotechnology for the development of better orthopedic implants”, *Journal of Biomedical Nanotechnology*, pp. 18-29.
6. E. Bekyarova, Y. Ni, E. B. Malarikey, V. Montana, J. L. McWilliams, R. C. Haddon, and V. Parpura (2005), Applications of carbon nanotubes in biotechnology and biomedicine, *Journal of Biomedical Nanotechnology*, pp. 3-17.
7. D. M. Ciombor, R. K. Aaron (1993), “Influence of electromagnetic fields on endochondral bone formation”, *Journal of cell biochemistry*, pp. 37-41.
8. D. M. Ciombor, R. K. Aaron (2005), “The role of electrical stimulation in bone repair”, *Foot Ankle Clinical*, pp. 579-593.
9. L. Cen, K. G. Neoh, and E. T. Kang (2002), “Surface functionalization of electrically conductive polypyrrole film with hyaluronic acid”, *Langmuir*, pp. 8633-8640.
10. C. Yao, V. Perla, J.L.McKenzie, E.B. Slamovich, T.J. Webster (2005), “Anodized Ti and Ti6AlV possessing nanometer surface features enhance osteoblast adhesion”, *Journal of Biomedical Nanotechnology*, pp. 68-73.
11. R. Dagani (1998), “Nanomania”, *Chemical and Engineering News*, pp. 70-77.
12. J. L. McKenzie, M. C. Waid, R. Shi, and T. J. Webster (2004), “Decreased functions of astrocytes on carbon nanofiber materials”, *Biomaterials*, pp.1309-1317.
13. T. J. Webster (2004), “Increased osteoblast adhesion on nanophase metals: Ti, Ti6A14V, and CoCrMo”, *Biomaterials*, pp. 4731-4739.
14. F. Kreupl, A. P. Graham, G. S. Duesberg, W. Steinhögl, M. Liebau, E. Unger and W. Hönlein (2002), “Carbon nanotubes in interconnect applications”, *Microelectronic Engineering*, pp. 399-408.
15. A. Rochefort, P. Avouris, F. Lesage, D. R. Salahub (1999), “Electrical and mechanical properties of distorted carbon nanotubes”, *Physical Review B,* pp. 824-830.
16. B. S. Harrison, A. Atala (2006), “Carbon nanotube applications for tissue engineering”, *Biomaterial*, pp. 1-20.
17. P.R. Supronowicz, P.M. Ajayan, K.R. Ullmann, B.P. Arulanandam, D.W. Metzger, R. Bizios (2002), “Novel current-conducting composite substrates for exposing osteoblasts to alternating current stimulation”, *Journal of Biomedical Materials*, pp. 499–506.
18. B. Zhao, H. Hu, S.K. Mandal, R.C. Haddon (2005), “A bone mimic based on the self-assembly of hydroxyapatite on chemically functionalized single walled carbon nanotubes”, *Chemistry of Materials*, pp. 3235–3241.
19. K. Besteman, J.O. Lee, F.G. Wiertz, H.A. Heering, C. Dekker (2003), “Enzymecoated carbon nanotubes as single-molecule biosensors”, *Nano Letters*, pp. 727–730.