Published in final edited form as:

J Med Biol Eng. 2018 April ; 38(2): 197–210. doi:10.1007/s40846-017-0312-7.

Effect of Collagen-Polycaprolactone Nanofibers Matrix Coating on the *In Vitro* Cytocompatibility and *In Vivo* Bone Responses of Titanium

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Abstract

The objective of this study was to improve the biomechanical performance of titanium (Ti) using a biocompatible electrospun nanofiber matrix. The study is based on the hypothesis that coating a Ti surface with a nanofiber matrix (NFM) made of collagen (CG) and polycaprolactone (PCL) electrospun nanofibers could increase the mechanical fixation of Ti/bone by improving the surface and cytocompatibility properties of Ti. This study prepared Ti samples with and without CG-PCL NFM coatings. This study determined the *in vitro* effects of each group of Ti samples on the surface topography and cytocompatibility (osteoblast cell adhesion, proliferation, mineralization and protein adsorption) properties. This study also determined *in vivo* interface shear strength and bone volume fraction of each group of Ti samples with bone using a rabbit model. This study found that the CG-PCL NFM coating on Ti improved the surface roughness, osteoblast cell adhesion, proliferation, mineralization and protein adsorption properties of Ti. *In vivo* studies found that interface shear strength of CG-PCL NFM-coated Ti/bone samples was significantly higher compared to those values of control Ti/bone samples (p value < 0.05) due to an increase in the amount of growth of the connective tissue joining the Ti implant. Therefore, the developed CG-PCL NFM coating technique should further be investigated for its potential in clinical applications.

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6. Conflict of interest

The authors have no conflicts of interest.

Keywords

Titanium; Bone; Interface Shear Strength; *In vivo*; Osseointegration; Cytocompatibility; Implant

1. Introduction

Titanium (Ti) alloys have been widely used in orthopedics and orthodontic surgeries as implants [1]. The differences in biomechanical performance between tissue-tissue and tissue-Ti implant interfaces are mainly due to the difference in contact geometry, material properties, and bone responses between the interface materials. Native tissue-tissue interfaces have mechanical interlocks with naturally occurring osteogenesis functions existing between the joining tissues [2]. In contrast, the Ti implant surface is inert and does not have any biological constituents that can regulate and enhance tissue-Ti implant-interface osteogenesis functions to mimic the characteristics of native tissue-tissue interfaces. There is an unmet need to increase the osteogenesis-stimulating factors to improve the osseointegration of Ti. Biological coatings made with nanofiber matrix (NFM) on implant surfaces can provide the necessary cell signaling information that can be communicated between implant and joining tissues for new bone generation at the tissue -Ti implant interfaces [3]. These cellular-level signals can play a central role in controlling cell fate and establishing tissue structure and function [3]. The objective of this study was the application of a novel electrospun NFM coating on a Ti implant to improve both biological and mechanical functions of Ti.

Electrospinning is a process by which fibers with micron- to nanometer diameters can be delivered by needle to a substrate from an electrostatically driven jet of polymer solution [4,5]. These fibers have a high surface area-to-volume ratio, which can be used to produce NFM for biomedical applications [6,5,7]. Polycaprolactone (PCL) nanofibers can be produced using an electrospinning process that is biocompatible and nontoxic [8,7]. The problem with the application of PCL electrospun nanofiber coating on Ti implant is the poor adhesion of PCL to the Ti surface. The adhesion of PCL to Ti can be improved by plasma oxidizing the Ti surface, then coating the Ti surface with CG followed by depositing PCL nanofibers on Ti, and coating the Ti surface again with CG [9]. CG is a scleroprotein, one of a family of proteins marked by low solubility in water [10]. In addition, CG is especially rich in the amino acid glycine, and it is the only protein known to contain a substantial proportion of hydroxyproline that plays versatile roles in cell metabolism and physiology [11]. There were two reasons to use CG with PCL. Firstly, CG is a biological adhesive material [12] that acts as a sealant for attaching PCL nanofibers onto the Ti surface. Secondly, CG can improve the hydrophobicity of PCL that can influence the adhesion of cells with PCL NFM [13,14].

This study was motivated by our previous observation that filling microgrooved-Ti implants with CG-PCL NFM coating significantly improved the mechanical stability and osseointegration of the Ti implant in a rabbit model of bone healing. The study showed that the mean shear strength values of Ti/bone were significantly higher for CG-PCL NFM coated grooved Ti samples (4.79 ± 0.39 MPa, $n = 6$) compared to grooved, uncoated Ti

samples (0.84 ± 0.3 MPa, $n = 6$) (p value < 0.05). Histomorphometric analyses showed that the amount of bone and tissue growth on CG-PCL NFM-coated Ti samples was higher than control. μ CT analyses showed that CG-PCL NFM coating influenced the bone density adjacent to Ti. In this study, we have evaluated the effect of CG-PCL NFM on the surface topography, cytocompatibility and mechanical response of non-groove Ti samples. The study was based on the hypothesis that deposition of NFM made with CG-PCL on a Ti surface could increase the mechanical fixation of Ti/bone by improving the surface and cytocompatibility characteristics of Ti. A thorough understanding of the CG-PCL coating structure (e.g. topography, thickness) and biomechanical function (cytocompatibility and mechanical responses) relationship at *in vitro* and *in vivo* conditions is necessary to evaluate the efficacy of CG-PCL NFM coating as a functional bio-coating for orthopedic/orthopedic implant [15]. Therefore, the first objective of this study was to evaluate the *in vitro* effects of CG-PCL NFM coating on the surface topography and cytocompatibility (osteoblast cell adhesion, proliferation, mineralization and protein adsorption) properties of Ti. The second objective of this study was to determine the effect of CG-PCL NFM coating on the *in vivo* mechanical fixation and bone volume fraction of Ti with bone using a rabbit model.

2. Materials and Methods

2.1. Materials

This study used 6Al-4V ELI (ASTM B 348 standard, grade 23, biocompatible) Ti alloy as the Ti implant, since it has better physical and mechanical properties than pure Ti [16]. A 9.565 mm diameter $\times$ 6 mm length 6Al-4V ELI Ti alloy round rod was used for *in vitro* studies, and a 2.22 mm diameter $\times$ 14 mm length 6Al-4V ELI Ti alloy round rod was used for *in vivo* studies. Both sizes of Ti rods were purchased from Titanium Metal Supply, Inc., Poway, CA. The NFM coating was composed of CG and PCL nanofibers. PCL nanofibers were prepared using PCL pellets (pellet size $\sim$ 3 mm, average Mn 80,000) and acetone (laboratory reagent 99.5%). Both PCL and acetone were purchased from Sigma Aldrich (Sigma-Aldrich Co. LLC., St. Louis, MO). Type I collagen (Corning.), which was extracted from rat tail, was purchased from Fisher Scientific.

2.2. Sample design

Two groups of Ti samples were produced for *in vitro* and *in vivo* studies. The Ti surface was polished in the first group (control Ti). CG-PCL NFM was deposited on the second group of polished Ti samples (NFM coated samples). A total of 6 samples (3 samples per group) were used for *in vitro* confocal surface characterization and cytocompatibility tests. This sample size is in agreement with literature to get statistical significance of the influence of a bio-coating on the *in vitro* cytocompatibility properties of an implant [17,18]. A total of 8 rabbits (4 rabbits per group) were used for *in vivo* mechanical and μ CT tests. Each rabbit received two of the same group of Ti samples, one Ti sample per leg. Four samples from random rabbit legs (left or right) were used for pull-out tension tests per group to get statistically independent samples for each group similar to Peter *et al.* [19]. The other implant on each rabbit was reserved for a separate study. Each mechanical test sample was μ CT scanned for the determination of bone volume fraction around the Ti implant.

2.3. Production of aligned electrospun nanofibers

Aligned PCL nanofibers were deposited between two parallel wires using an electrospinning setup. The details of PCL electrospun nanofiber fabrication can be found in Khandaker *et al* [20]. In short, PCL pellets (7.69 wt%) were mixed with acetone in an ultrasonic mixer (Sonics & Materials, Inc., model # Vibra-cell VCX 130). The sonication process was carried out at approximately 60°C for 30 minutes. The solution was poured into a glass syringe on an infusion pump (Harvard Apparatus, mode # PHD ULTRA) for PCL fiber production. The PCL solution was ejected from the glass syringe through an electrically-charged needle (23G blunt needle, 1 in length, model # BX 25). The needle was positively-charged by a high voltage power source (Gamma High Voltage Research, Inc., model # ES 30 series) and two parallel wires were negatively-charged. Aligned PCL fibers were collected between the two parallel wires. These fibers were used for coating the *in vitro* and *in vivo* NFM test samples, which were prepared by a set of steps as schematically represented in Fig. 1.

2.4. Sample preparation

2.4.1. *In vitro* test samples—Ti samples for *in vitro* studies were mirror polished using a Buehler Ecomat grinder/polisher to remove scratches from cutting and to have a uniform surface finish for all test samples. A three-step polishing technique, as recommended by Buehler [21], was used to polish the Ti surface. The first step was to polish using Buehler CarbiMet 2 Abrasive Discs. While polishing, 9µm MetaDi Supreme Diamond Suspension was sprayed on the contact surface of the Ti rod and the polishing disc. The second polishing step used an Ultra Pad with 0.05µm MetaDi Supreme Diamond Suspension from Buehler. The last polish was performed using MicroCloth and MasterPrep Alumina. After each step, Ti rods were cleaned by ethanol in an ultrasonic cleaner and then sterilized by autoclaving. Ti rods without further treatment were considered as control Ti samples.

Collagen solution was prepared by mixing 2.3 microliters of type I collagen with 0.23 microliters of acetic acid (0.02 M) and 195 microliters of deionized water in a vortex mixer. Ti samples were exposed to plasma O₂ for 5 minutes in a Zepto low pressure reactive ion etching system (Frequency: 40 kHz, power 30 watt) to increase the attachment of CG to the Ti surface [22]. The top face of the Ti rod was covered with the CG solution (~ 70 microliters). Multilayers of aligned PCL electrospun nanofibers (~0.6 milligram) were deposited on the top face of the Ti by repeating forward-upwards lifting of the Ti rod six times along the parallel wire collectors. The sample was dried in a UV chamber. Finally, the CG solution coating on Ti was applied again and dried to prepare the NFM coated Ti samples.

2.4.2. *In vivo* test samples—All groups of *in vivo* Ti samples were circumferentially polished up to 8 mm from one end using a drill machine check and gripper. Ti wires were polished for a fixed time (6 minutes) in order to have uniform surface morphology for all test samples. Ti wires were polished, then cleaned and sterilized according to the same method described in the previous section. Ti wires without further treatment were considered as control samples.

To prepare NFM coated Ti samples, Ti wires were soaked with the CG solution (~10 microliters) around the circumference after plasma oxidation. Aligned PCL electrospun nanofibers (~0.2 milligram) were deposited along the circumference of the Ti by manually rotating the Ti wires 6 times and drying in a UV chamber. Ti samples were covered with nanofibers up to 8 mm from the polished end, since the Ti/bone contact area was created for all *in vivo* test samples only up to 8 mm. The non-fiber coated area of the Ti samples (~4 mm) was used for gripping samples for the mechanical tests. Finally, CG solution coating was applied to the Ti again and dried to prepare the NFM coated Ti samples. The samples were kept at 4°C until implantation in the rabbit femur.

2.5. Animal surgery

Each group of Ti samples was implanted immediately proximal to the lateral femoral condyle of 4–6 week-old New Zealand White Rabbits (NWR) at the OUHSC animal care facility. The protocol for animal study was approved by the OUHSC IACUC committee. A total of 8 NWR were used for this study. Rabbits were anesthetized with ketamine and xylazine by intramuscular injection and transported to the surgical preparation room. Rabbits were given buprenorphine and oxytetracycline. The entire circumference of both rear legs proximal and distal to the distal femur of the rabbits was shaved. Then each leg was sterilized with a chlorhexidine scrub alternating with alcohol. The rabbits were intubated with a 3mm ID endotracheal (ET) tube. Isoflurane was administered with oxygen via the ET tube. A final surgical prep was then performed. 0.5 ml of 0.5% lidocaine was injected subcutaneously at the incision site. Surgery started once the rabbit was completely at a surgical plane of anesthesia.

A 2.5 cm curvilinear midlateral skin incision was made starting at the distal end of the body of the femur and extending distally to just below the level of the patella. The fascia lata was then excised in line with the skin incision. The vastus lateralis muscle was then retracted cranio-medially to expose the lateral condyle and the joint capsule opened sharply. A drill bit (size 2.26 mm) was used to drill a 6–7 mm depth hole perpendicularly into the lateral condyle and then the Ti wire sample was hand-pushed into the hole. The reason for selecting a size 2.26 mm drill bit was to create a clearance hole for the implantation of the Ti sample. The joint capsule, muscle layers, and subcutaneous tissue were then sutured with 4–0 vicryl in a continuous pattern, and the skin was closed with a subcuticular pattern. X-ray images of the implantation sites were conducted immediately after surgery to measure the depth of the implant inside the bone (Fig. 2). All rabbits received buprenorphine post-operatively for 3 days and were closely monitored for food and water intake, fecal output, and healing properties.

2.6. Experiments

2.6.1. General observation of test samples—The state of NFM coating on the *in vitro* test samples was investigated before and after 3 weeks of cell culture on Ti samples for the mineralization assay. The samples were viewed microscopically before staining to evaluate the fiber existence, configuration, and attachment on the Ti surface. The Ti/bone sample status for the *in vivo* test samples was also investigated before and after the mechanical tests. Any callus formation and extraosseous ossification around the implant was

examined after cleaning the soft tissue from the bone before mechanical tests were performed.

2.6.2. Surface topography—Surface topography is an important parameter that plays a significant role in implant-bone adhesion. The influence of NFM treatment to surface morphology was evaluated using a Leica DCM8 surface metrology instrument under 50x bright field confocal control conditions. Scanning was conducted over 350.622 μm length $\times$ 263.934 μm width $\times$ 10.7763 height for all samples. Topography images were compared for three control and NFM coated *in vivo* Ti samples. Thickness of the PCL nanofibers was measured by line scanning across the fiber from the three NFM coated samples.

2.6.3. Cell adhesion, proliferation, mineralization, and protein adsorption tests on Ti samples—Rat osteoblast cells (R-OST-583; Lonza) were cultured at log phase growth in standard culture conditions (37°C in a 5% CO₂ incubator on tissue culture dishes) using DMEM/high glucose + 5% FBS and 1% ABAM (Sigma Chemical). Cells were dissociated using 1x trypsin/EDTA solution (Sigma Chemical) for 5 minutes at room temperature, followed by serum inactivation. Cells were counted using a hemocytometer, collected by centrifugation and re-suspended at a concentration of 35,000 cells per 400 microliters of growth media. Cell adhesion, proliferation, differentiation and protein adsorption tests on Ti samples were conducted according to the method as described in detail in Khandaker *et al* [17]. In short, osteoblast cells were seeded at a density of 35,000 cells/ml on each group of Ti samples in a custom-made silicone well-plate. Cells were then cultured for 48 hours to allow cell adhesion and proliferation on the Ti surface. Parallel samples similar to those tested for adhesion and proliferation were cultured for 3 weeks and prepared for immunostaining to determine hydroxyapatite mineralization and osteonectin adsorption. A Click-iT® EdU stain was used to evaluate cell adhesion and proliferation assay for each sample according to vendor's protocol [23]. This 48-hour assay involved the addition of EdU, or 5-ethynyl-2'-deoxyuridine, to each well after the initial 24 hour incubation. The EdU was a modified thymine nucleotide that contained a terminal alkyne. After a total of 48 hours, the cells were fixed with paraformaldehyde and stained with Alexa-488. The terminal alkyne in the EdU reacted with the azide in Alexa-488, which caused the proliferated cells that incorporated the EdU tag to fluoresce green under fluorescent microscopy. An OsteoImage™ mineralization assay kit from Lonza was used according to vendor's protocol. For the protein adsorption test, osteonectin was used as the primary antibody (clone AON-1; Developmental Studies Hybridoma Bank) and goat anti-mouse rhodamine (red) was used as the secondary antibody. For proliferation, mineralization, and protein adsorption tests, nuclei were counterstained with DAPI stain (blue). The qualitative and quantitative measurements of cell viability on the treated Ti surfaces were conducted from images captured with an Olympus DP72 camera and CelSens software. Cell adhesion on the surface of all types of Ti samples was analyzed for the qualitative measurement of cell viability. The number of cells adhered and the number of cells proliferated after adhesion to each sample were determined from the captured images using the ImageJ software program (<http://imagej.nih.gov/ij/>). Cell densities on NFM-coated Ti samples were compared with the control Ti samples for the quantitative measurement of cell adhesion and percentage of proliferation. The ratio of mineralized and osteonectin stain

area over total area of the image field was used to compare mineralization and osteonectin activities between control and NFM coated Ti samples, respectively.

2.6.4. Static tests on Ti/bone samples—Rabbits were euthanized 6 weeks after surgery. Soft tissues were carefully dissected off the bone. The Buehler saw machine was used to trim the Ti-bone samples to adequate size for static tests. The samples were kept moist during the preparation by soaking with saline. A custom-made fixture was used to permit coaxial alignment of the implant in the direction of pull-out force (Fig. 3). Since it requires a low force to break the Ti/bone interface, a 10 lb force sensor (Futek LCM 300) and a displacement sensor (Microstrain DVRT) were secured with the grippers of a Test Resource Universal Testing machine (UTM) for the high precision acquisition of force and displacement data from the static test. One side of the force sensor was secured in the bottom gripper of UTM via a threaded stud. The other side of the force sensor was secured in an aluminum cup, referred to as the cement holder in Fig. 3. A low-viscosity acrylic bone cement (BioMedtrix veterinarian bone cement) was poured into the cement holder. The Ti/bone sample was carefully fastened in the top gripper and slowly lowered to embed the sample in the cement. The displacement sensor was secured with the grippers using magnetic clamps. After an hour of cement curing, a pull out tension test was conducted on the sample at room temperature with a steady speed of 0.3 mm/min [24] until breakage of the Ti from the bone. The maximum pull-out force was measured and maximum shear strength was calculated from the ratio of the force at the point of implant breakage from the bone versus the surface area of Ti in contact with bone. The Ti/bone samples were stored in a freezer for μ CT analysis.

2.7. μ -CT

Eight Ti samples were scanned by μ -CT after the pull-out tension tests to examine the bone volume fraction and bone density around the implant site. A Gamma Medica X-PET XO machine was used at 80 KeV with slices every 50 μ m. A threshold of 1400+ was selected to delineate the bone using the AMIRA 5.6 software, then the edges were smoothed for the 3D volume, using a size of 3. A column with radius 2.25 mm centered on the implant hole was selected (Fig. 4a). This column was segmented into three rings with radii of 2.25–1.75, 1.75–1.25, 1.25–0.75 mm and a single column with radius 0.75 mm (Fig. 4b). These were each further segmented into 4 sub-regions using thresholds: Cortical bone: 2000+, Trabecular bone 1400–1999, Flesh: 500–1399, and Empty space: <500. These values were determined by scanning the pre-implanted rabbit femur before surgery (Fig. 4c). Figure 4d shows the bone volume belonging to each of the concentric cylindrical rings from the center axis of an implant hole. The total bone volume fraction in the 150 μ m of bone around the implant was calculated as the ratio of the number of voxels belonging to total bone (cortical plus trabecular) versus the total number of voxels belonging to the total volume of the cylindrical ring column within radii of 1.25 mm. Cortical bone volume fraction in the same area was calculated as the ratio of the number of voxels belonging to cortical bone versus the total number of voxels belonging to the total volume of the cylindrical ring column within radii of 1.25 mm.

2.8. Statistical Analysis

To determine whether there is a significant difference in the means of the roughness variables of the control and NFM coated Ti groups, independent samples *t*-tests were performed assuming unequal variances. For all other variables, one-factor analysis of variance (ANOVA) with subsampling was performed. If necessary, the ANOVA was followed by multiple comparisons with Tukey's adjustment. All statistical analysis was done in SAS v. 9.3 using proc mixed. All data were presented in the literature as mean $\pm$ standard error of the mean (SEM) (number of samples (*n*)).

3. Results

3.1. Physical state of Ti samples

A clear difference of surface topography between the control and NFM coated Ti samples was observed (Fig. 5a and Fig. 5b). Attachment of aligned PCL fibers was clearly visible for *in vitro* and *in vivo* NFM coated Ti samples. After *in vitro* biocompatibility tests were conducted on NFM coated Ti surfaces, results show the attachment of PCL NFM coating after 3 weeks of cell culture on the Ti surface. It is important to mention that during the cell culture period, the coating material had to withstand the regular cell culture medium application and aspiration forces every 3 to 5 days. *In vivo* mechanical tests showed all Ti samples bonded with bone and none show the existence of fiber on the Ti surface after the 6 weeks of implantation.

3.2. Surface topography

A clear visible difference in surface topography was observed from confocal microscope images between the Ti and NFM coated Ti samples (Fig. 6). Significant differences of roughness parameters, except maximum peak height, were observed between the sample groups (Table 1). This result suggested the surface texture and porosity changed significantly due to the NFM treatment. Multiple fiber overlapping was observed in NFM coated Ti samples (Fig. 6b). Figure 7 shows the line profile along a path that crossed an independent fiber site, which was circled in Fig. 6b. A 0.29 μm observed difference in the average depth between non-fiber coated and fiber coated sites was predicted to be the thickness of the nanofiber. The thickness of fiber from confocal images of NFM coated samples was found to be $0.272 \pm 0.004 \mu\text{m}$ ($n = 3$).

3.3. Cytocompatibility properties

Cell adhesion and proliferation successfully occurred on the surface of all types of Ti samples as shown in Fig. 8. Both samples demonstrated adhered, non-proliferating cell nuclei (blue color DAPI stained) and proliferating cell nuclei (green color click-Alexa 488 stained). Cells grew along with fibers more readily in NFM coated Ti samples and with increased cluster distribution along the surface of the fibers on the Ti surface compared to control. There was a significant difference in the mean cell densities (combined blue and green nuclei) of the control and NFM treated Ti sample groups ($F_{1,4}=4.50$, p value=0.04), but there was no significant difference in the mean percentage of cell proliferation

($F_{1,4}=1.86$, p value=0.247) as shown in Fig. 9. These results suggest that NFM coating may have a positive influence on the *in vitro* osseointegration with the Ti surface.

Cell mineralization and protein adsorption successfully occurred on the surface of all types of Ti samples as shown in Fig. 10 and Fig. 11. Both samples demonstrated adhered cells (blue nuclei) and hydroxyapatite mineralization from the cells (green stained). There was no significant difference in the mean amount of mineralization (total green stained area $\times$ 100%/area of image field) of the control and NFM treated Ti sample groups (p value=0.287) (Fig. 12). Both samples demonstrated adhered cells (blue nuclei) and presence of osteonectin from the cells (red stained). There was a significant difference in the mean amount of osteonectin (total red stained area $\times$ 100%/area of image field) between the control and NFM treated Ti sample groups (p value=0.001) (Fig. 12).

3.4. Mechanical tests

A sudden increment of load and fracture of Ti from bone was observed for all specimens. Such high initial load was higher for NFM treated samples compared to the control samples. A negligible amount of displacement before the initiation of crack at the interface was found for both sample groups. The experimental parameters for the calculation of the mean interface shear strengths for the control and NFM coated groups are displayed in Table 2. There was a significant difference in the mean fracture strengths of the groups ($F_{1,4}=11.93$, p value=0.024). These results suggest that NFM treatment has a positive influence on the mechanical fixation between Ti and bone interfaces.

3.5. μ CT analysis

The difference of mean cortical bone volume fractions between control and NFM coated Ti samples was statistically significant (Table 3) ($F_{1,4}=12.31$, p value=0.022), but no significant difference in the mean total bone volume fractions between control and NFM coated Ti samples was observed ($F_{1,4}=2.71$, p value=0.15). This result suggests that the NFM coating treatment on Ti created a higher amount of cortical bone tissue at the interface compared to control that might lead to higher interface shear strength for NFM treated samples compared to control.

4. Discussion

A clear topographical difference was observed between control and NFM coated samples from the confocal microscopy images. There was a variable amount of fiber deposition, alignment, and diameter of the fiber along the NFM coated Ti surface (Fig. 6b). This variability happened due to the variation of the fiber production and manual collection process. The topographical difference among the NFM coated Ti samples led to a higher amount of variance (represented by SEM) in roughness parameters for NFM coated Ti samples than the control samples (Table 1). To reduce the effect of the fiber topography on the *in vitro* cytocompatibility and *in vivo* mechanical test results for the NFM coated Ti samples, fibers were deposited on the parallel wire collectors for the same time period (30 seconds) under the same fiber production conditions (PCL solution viscosity, DC voltage and solution flow rate). In addition, the same number of fiber layers (six total layers) were

applied to each NFM coated Ti sample. An automated fiber collection process from the parallel wire collectors was required to further minimize the topographic variation effect on the cytocompatibility and mechanical test results of NFM coated Ti samples.

CG-PCL NFM coated Ti samples demonstrated better biological and mechanical responses than non-treated Ti samples. Over the three-week period of *in vitro* culture, osteoblasts were able to persist, proliferate, and differentiate on the Ti substrate in the presence and absence of NFM. The adhesion and proliferation tests results of control Ti samples were similar to our previously published report [17]. The percentage of proliferation in control versus NFM coated Ti was not significant. This suggests that the rate of proliferation was constant in both treatments. However, more cells were clearly present in clusters for the NFM coated Ti compared to control (Fig. 8). Two hypotheses can be provided for this response: 1. When cells initially were plated, they adhered better to the NFM and therefore more cells were present initially; or 2. Cells were dying at a greater rate in Ti compared to NFM. Neither of these hypotheses was tested here but would be interesting to study in the future.

Osteoblast cell differentiation was demonstrated by hydroxyapatite (Fig. 10) and osteonectin expression from fluorescent-stained images (Fig. 11). While osteonectin expression increased with NFM treatment, no mineralization increase occurred in the NFM group (Fig. 12). It is possible that when osteoblasts are provided with a CG substrate, they downregulate their own production of CG [25]. The provided CG (rat tail type I) might be suboptimal for outside-in signaling compared to self-secreted and MMP-matured CG, thereby delaying mineralization. Numerous other molecules are involved in the matrix maturation process that may also be affected by exogenous CG deposition [26].

This study found low fracture load in both control and NFM coated *in vivo* test samples. This happened due to the fact that this study created at least a 40µm-clearance between the Ti and bone surface during surgery for both sample groups, which was filled with connective tissue within 6 weeks of the implantation period. Such clearance was created to measure the effect of NFM on *in vivo* mechanical fixation and growth of connective tissue (quantified by bone volume fraction). The values of shear strength of Ti/bone for both control and NFM coated Ti samples confirmed the development of the connective tissue between Ti and bone. The cortical bone volume fraction adjacent to the implant and interface shear strength values were higher for NFM coated samples than control (p value <0.05). Since bone volume fraction and interface shear strength are directly correlated in the vicinity of the implant [19], therefore, we can conclude that an increased amount of cortical bone tissue in the vicinity of the implant led to the higher shear strength of Ti/bone for NFM coated samples compared to control.

In this study, we observed increased cytocompatibility properties (adhesion, proliferation, and protein adsorption) of NFM coated Ti implants compared to Ti. This is due to the fact that higher cell functions were created via better cell signaling arising from the cell-cell contact and the cell-NFM components in the case of the NFM coated Ti samples [27]. Cell signals depend upon physical (micro- or nano-structured surface topography, composition of NFM) and chemical (presence of amino acid) properties [28,29]. There exist differences of physio-chemical properties between control and NFM coated Ti samples. The CG blended

PCL nanofibers on Ti lead to different physical characteristics viz. porosity, density due to distribution of the PCL fiber sandwiched in between two layers of CG coating. CG in the NFM coating has the characteristic proline-rich domain, where Gly is found in every third residue with tripeptide repeats of $-(\text{Gly-X-Y})_n-$ in which proline (x) and hydroxyproline (Y) hold 20% of the amino acid composition [13]. PCL in the NFM coating created a larger surface area to have more cell binding sites. Also PCL NFM can absorb higher cell membrane-bound proteins or minerals, thus favoring cytocompatibility properties for NFM coated Ti samples compared to control.

In this study, we have observed increased mechanical fixation (quantified by interface shear strength) of NFM coated Ti implants compared to Ti. This is due to the fact that *in vivo* osseointegration of the implant with bone depends on implant surface biocompatibility [30], which was found to be higher for NFM coated Ti samples than Ti from the *in vitro* cytocompatibility assays. The implant - tissue interface was surrounded by nano composite structures in NFM coated Ti samples. These nanostructures were formed by CG and PCL biomolecules organized in geometrical arrangements with favorable porosity for cellular function. The higher cell interaction between NFM coated Ti and bone might lead to a higher amount of connective tissue created between the NFM implant and bone than between control Ti and bone. The μCT analysis results validated the previous argument where we found a higher amount of bone volume fraction of NFM coated samples than control samples.

To our knowledge, this is the first study to evaluate the effect of CG-PCL NFM coating treatment on the *in vitro* cytocompatibility on Ti using osteoblast cells and the *in vivo* mechanical stability of Ti implants using a rabbit model. Albu *et al.* [31] reviewed micro and nanofabrication of different CG based biomaterials that led to different physical and biocompatibility characteristics. Zhang *et al.* [32] conducted *in vitro* characterization of surface biocompatibility of scaffolds made of electrospun PCL, CG, and electrospun PCL-CG nanofibers for the growth of human dermal fibroblasts. The previous authors' study concluded that individual PCL nanofibers coated with CG tend to resemble the natural NFM rather than the porous CG coating or the pristine PCL nanofibers. Therefore, fibroblasts have a greater propensity to interact well with the former than the latter, suggesting a greater ability to construct complex sets of cellular interactions as biomimetic nanofibers in engineering tissues. We have found in this study that osteoblasts have a greater propensity to attach well with the fiber matrix compared to non-coated, and proliferate at the junction of the two crosslinked fibers. Zankovych *et al.* [33] found that the maximum shear strength of Ti/bone interface in a rat model after 8 weeks of surgery was 0.15 to 0.6 MPa. These results are in agreement with the maximum shear strength of Ti/bone samples for control Ti found in our study (0.18 ± 0.05 MPa).

This study used Ti as the control sample instead of CG coated Ti due to the surface characteristics of Ti. Uniform coating of CG on Ti is not possible when only CG was applied to Ti. Concentric and non-uniform adhesion of CG on Ti was observed during the splash of CG on Ti. This is due to the high contact angle and low wettability of CG on the polished Ti surface. Combined application of plasma oxidation and PCL NFM was utilized in this study to spread CG uniformly on the Ti surface. Further investigation is required to measure the

contact angle, wettability, and adhesion of Ti, CG coated Ti and CG-PCL NFM coated Ti samples to understand the effect of changing the PCL and CG ratio on the topography of NFM on Ti.

This study is limited to the cytocompatibility tests of non-treated and NFM coated Ti samples. The cell adhesion density of PCL coated Ti, CG coated Ti, and CG-PCL coated Ti was explored in our earlier study [34]. This study found poor attachment of PCL with Ti and negligible cell attachment with PCL. The study found that cell adhesion densities of CG-PCL coated Ti samples (522.27 ± 25.87 , $n = 30$) were significantly higher than CG coated Ti samples (405.17 ± 20.58 , $n = 30$) (p value < 0.05). Cells grew arbitrarily in CG coated Ti, while cells grew more readily along the fiber direction and increased cell clustering along the surface of the fibers on Ti surface similar to this study. Therefore, CG-PCL NFM coated Ti samples were further investigated in this study to evaluate the effect of CG-PCL NFM coating on other cytocompatibility properties on Ti (proliferation and differentiation). Further study is required to show concentration effect of CG and PCL in the NFM on the cytocompatibility properties of Ti.

This study is limited to the static pull-out tension tests on different groups of Ti/bone samples. The strain rate used for these studies was 0.3 mm/min or 0.005 mm/sec. This strain rate was selected due to the fact that fractures occur in bone at strain rates of between 0.3 and 1 [24]. Since a large increment of load occurs before fracture during the mechanical tests, the study can collect more data near the fracture stage using the 0.3 mm/min strain rate than higher strain rate. This is important for recording the mechanical responses (load and displacement) of the implant-bone joint, since small forces with negligible displacement were required for sample breakage.

The novelty of this study is the *in vitro* and *in vivo* evaluation of CG-PCL NFM coating treatment on a Ti alloy for improving its biological and mechanical responses. Specifically, this study measured for the first time the effect of the CG-PCL NFM coating treatment on the *in vitro* biocompatibility of a Ti alloy (Ti-6Al-4V ELI) and *in vivo* interface fracture strength of Ti/bone. This article contributes to the biomaterials community by providing increased understanding of how the biomechanical functions of the Ti implant can be improved by CG-PCL NFM coating. In particular, this research shows Ti implants can be coated with CG-PCL NFM coating to increase the cytocompatibility properties of Ti, thereby improving osseointegration of the implant. This study provided proof of concept data for the development of our novel surface modification approach for orthopedic implants. In this approach, micro size grooves were fabricated on Ti implants and CG-PCL NFM was filled inside the micro size grooves. Since the NFM adhered inside the Ti grooves, NFM coating does not withstand the shear force during the *in vivo* insertion of Ti implant.

Surface blasting and chemical etching are some of the common approaches that generate mechanical interlock to improve the integration between an implant and the bone tissue [30]. Plasma-surface modification techniques such as plasma spray and plasma immersion have the ability to selectively enhance surface biomechanical characteristics by ion deposition [37]. Nanofiber bio-coating techniques as developed in this research can be applied on the Ti surface as a supplementary processing approach without greatly affecting the positive

benefits from other surface etching or deposition techniques. The major advantage of our approach is that PCL nanofibers, due to their unique surface-to-volume ratio, can be carriers for local delivery of biomolecules like hydroxyapatite and magnesia [13]. Such functional bio-coating could improve mechanical stability and osteogenesis functions of bone adjacent to an implant. Therefore, our developed technique can be superior in comparison to other biomaterial coatings.

5. Conclusion

This study found statistically significant improvements on the osteoblast cytocompatibility properties of Ti and mechanical fixation of Ti with bone for CG-PCL NFM treated Ti samples compared to the non-treated Ti samples. In addition, this study observed that CG-PCL NFM coating on Ti positively influences the volume of the new cortical bone growth around the Ti implant. Since the CG-PCL NFM coating method developed in this study improved the physico, biocompatibility, and mechanical fixation of Ti, it can be concluded that Ti surface modifications by CG-PCL NFM coating favor *in vivo* bone formation that leads to improved implant union with bone. The combined application of microgrooving and filling microgrooves with CG-PCL NFM may be applied in orthopedic or orthodontic implants.

Acknowledgments

This publication was made possible by Grant Number 5P20GM103447 from the NIH and on-campus faculty grant and CURE-STEM awards from the College of Math and Science and the University of Central Oklahoma Office of Research and Grants. The AON-1 osteonectin antibody developed by John D. Termine was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242. Special acknowledgment to Andria Hedrick, Research Imaging Facility at the College of Pharmacy in the University of Oklahoma Health Science Center, for μ CT analysis support.

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Figure 1.
Steps for fabrication of NFM coated Ti samples for *in vitro* and *in vivo* studies.

Figure 2.
X ray image of an implanted Ti in a rabbit femur.

Figure 3.

Mechanical test on a Ti/bone sample in a UTM machine. The picture shows the connection of a high precision load cell and displacement sensors with the UTM for the accurate measurement of load-displacement data from the pull-out tension test using the UTM. Right side image shows the pouring of low viscous bone cement for the anchorage of a Ti/bone sample in the UTM machine grippers.

Figure 4.

Process to find the bone volume fraction from a selected region of interest (ROI) of a μ -CT image: (a) column selection around the center of the implant hole; (b) concentric ring creation around the hole of the implant; (c) determination of CT threshold values to identify different types of bone tissue from a CT scan image of a rabbit femur bone before surgery (without implant). The CT scanning was conducted around the same location where the implant was inserted. (d) Use of density thresholds to assign portions of column to the specific bone tissue to generate 3D volume of the specified ROI.

Figure 5.

The stereomicroscope images of a fabricated *in vitro* samples that were used for the osteoblast cytocompatibility tests: (a) control and (b) NFM coated Ti. The stereomicroscope images of a fabricated *in vivo* samples that were used for the mechanical and μ CT tests: (c) control and (d) NFM coated Ti.

Figure 6.
Confocal topography microscope images of (a) control and (b) NFM coated Ti samples.
Both images were captured at 50x magnification.

Figure 7.
Confocal line scan depth profile across a fiber (inside the circle in Figure 7b).

Figure 8. A representative fluorescent stained image (100x magnification) from adhesion and proliferation assays showing adhered non-proliferating (blue color) and proliferating cells (green color) of control (top) and NFM coated (bottom) Ti samples. The length of the scale bar in each image is 100 µm.

Figure 9. Mean cell adhesion density ($\pm$ SEM) and percentage of cell proliferation ($\pm$ SEM) for the control and NFM coated Ti groups. Data presented $n=27$ for both samples. Note: * $p<0.05$ (compared to control).

Figure 10.

A representative fluorescent stained image (100x magnification) from mineralization assays showing cells nuclei (blue color) and released hydroxyapatite (green color) from cells of a control (top) and NFM coated (bottom) Ti samples. The length of the scale bar in each image is 50µm.

Figure 11.

A representative fluorescent stained image (100x magnification) from mineralization assays showing cells nuclei (blue color) and released osteonectin (red color) from cells of a control (top) and NFM coated (bottom) Ti samples. The length of the scale bar in each image is 50µm.

Figure 12.

Mean amount of mineralization ($\pm$ SEM) and mean amount of osteonectin ($\pm$ SEM) for the control and NFM coated Ti groups. Note: * $p < 0.05$ (compared to control).

Table 1

Roughness Parameters of Ti samples. Values are given as mean $\pm$ SEM ($n = 3$ each).

Parameter Description	Control	NFM coated	<i>t</i>	<i>p</i> -value
Root Mean Square Roughness, S_q (μm)	2.32 ± 0.19	$5.55 \pm 0.69^*$	4.51	0.036
Max Peak Height, S_p (μm)	3.56 ± 0.30	11.65 ± 2.82	2.85	0.102
Max Valley Depth, S_v (μm)	6.68 ± 0.80	$17.14 \pm 1.38^*$	6.56	0.006
Max Height of Surface, S_z (μm)	10.24 ± 0.51	$28.79 \pm 2.26^*$	8.01	0.011
Average Roughness, S_a (μm)	1.96 ± 0.15	$4.62 \pm 0.63^*$	4.09	0.046

* Significantly greater than control (p -value <0.05).

Table 2

Summary statistics for *in vivo* pull out tension test data by group. Values are given as mean $\pm$ SEM ($n = 4$ each).

Parameter Description	Control	NFM coated	<i>F</i>	<i>p</i> -value
Height of implant in contact with bone (mm)	7.90 $\pm$ 1.16	6.01 $\pm$ 0.54	2.18	0.206
Implant/bone contact area (mm ²)	54.73 $\pm$ 8.01	41.56 $\pm$ 3.75	2.22	0.203
Fracture load (N)	9.89 $\pm$ 1.93	17.39 $\pm$ 1.77	8.23	0.054
Shear strength of Ti/bone (MPa)	0.18 $\pm$ 0.05	0.43 $\pm$ 0.05 *	11.93	0.024

* Significantly greater than control (p -value<0.05).

µCT analysis of cortical bone amount in the 150 µm of bone around the implant. Values are given as mean ± SEM ($n = 4$ each).

Table 3

Parameter Description	Control	NFM coated	F	p-value
Scan depth	8.91 ± 0.83	9.14 ± 0.78	0.04	0.866
Total bone volume	20.54 ± 1.98	25.05 ± 1.90	2.70	0.151
Cortical bone volume	6.76 ± 0.94	11.42 ± 1.12 *	10.14	0.020
Total bone volume fraction	0.61 ± 0.06	0.75 ± 0.06	2.71	0.150
Cortical bone volume fraction	0.24 ± 0.04	0.38 ± 0.02 *	12.31	0.022

* Significantly greater than control (p -value<0.05).