Published in final edited form as:

Polym Adv Technol. 2019 May ; 30(5): 1205–1215. doi:10.1002/pat.4553.

Insulin immobilized PCL-cellulose acetate micro-nanostructured fibrous scaffolds for tendon tissue engineering

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Abstract

Use of growth factors as biochemical molecules to elicit cellular differentiation is a common strategy in tissue engineering. However, limitations associated with growth factors, such as short half-life, high effective physiological doses, and high costs, have prompted the search for growth factor alternatives, such as growth factor mimics and other proteins. This work explores the use of insulin protein as a biochemical factor to aid in tendon healing and differentiation of cells on a biomimetic electrospun micro-nanostructured scaffold. Dose response studies were conducted using human mesenchymal stem cells (MSCs) in basal media supplemented with varied insulin concentrations. A dose of 100-ng/mL insulin showed increased expression of tendon markers. Synthetic-natural blends of various ratios of polycaprolactone (PCL) and cellulose acetate (CA) were used to fabricate micro-nanofibers to balance physicochemical properties of the scaffolds in terms of mechanical strength, hydrophilicity, and insulin delivery. A 75:25 ratio of PCL:CA was found to be optimal in promoting cellular attachment and insulin immobilization. Insulin immobilized fiber matrices also showed increased expression of tendon phenotypic markers by MSCs similar to findings with insulin supplemented media, indicating preservation of insulin bioactivity. Insulin functionalized scaffolds may have potential applications in tendon healing and regeneration.

Keywords

cellulose acetate; growth factor alternative; insulin; micro-nanofibers; polycaprolactone; tendon; tissue engineering

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SUPPORTING INFORMATION

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1 | INTRODUCTION

Tendon injuries account for roughly 50% of the 33 million musculoskeletal injuries that occur in the United States annually.¹ Current strategies involving the use of allografts and autografts to treat tendon injuries are the gold standard, but still provide only suboptimal healing with minimal functional gains.² Tissue engineering has arisen with the aim to promote better healing and regeneration of musculoskeletal tissues through use of cells, biomaterials, and biochemical factors.^{3,4}

The application of growth factors as biochemical signals to elicit cellular differentiation is a common strategy in tissue engineering. Often growth factors are added to differentiation media to promote lineage specific differentiation of stem cells. However, there still remains a lack of standardized differentiation media for tendon. Various growth factors have been shown to support tendon differentiation.^{1,2,5} Insulin-like growth factor-1 (IGF-1) has been extensively researched for its ability to encourage cell proliferation, inhibition of cell apoptosis, and anabolic effects on musculoskeletal tissues.^{6–10} Specifically, IGF-1 has been identified to be involved in all phases of tendon healing that include inflammatory, proliferative, and remodeling stages.¹¹ During the inflammatory stage, RNA and protein levels of IGF-1 are upregulated indicating a role of IGF-1 during the early stages of healing.^{12,13} Mitotic effects of IGF-1 in tendon cells and tendon animal models have also been well documented.^{14,15} IGF-1 has also been observed to increase collagen synthesis.^{8,10,16} For example, injection of IGF-1 was shown to increase collagen content near the injection site.¹⁰ Additionally, application of IGF-1 has also been demonstrated to increase tendon markers.^{8,10} However, limitations associated with growth factors such as high costs and short half-life have prompted the search for growth factor alternatives, such as small molecules, growth factor mimics, peptides, or other proteins.^{17,18}

IGF-1 is a member of the insulin superfamily that includes insulin and IGFs. IGF-1 is named as such due its homology to insulin protein. Insulin and IGF-1 share 50% of their amino acid sequence, and the intracellular domains of their respected receptors share 84% homology.¹⁹ Insulin has been shown to bind to IGF-1 receptors²⁰ due the similar structure of both insulin and IGF-1 and their receptors.^{21–23} The homology between insulin and IGF-1 and their receptors in terms of structural composition has motivated various comparative studies,^{7,22,24} which have shown similar effects of insulin on tissue growth.^{25,26}

The analogous structures of insulin and IGF-1, as well as the observed overlapping effects of insulin, makes insulin an attractive candidate as an alternative for IGF-1. Since IGF-1 is homologous to its namesake insulin, it was hypothesized that insulin, such as IGF-1, may aid in tendon healing and promote tendon regeneration. Moreover, insulin may prove to be a cost-effective component in the creation of a tendon differentiation media.

Insulin, at microgram concentrations, has been applied towards cartilage differentiation^{25,27,28}; however, Mazzocca et al reported that mesenchymal stem cells (MSCs) in 2D cultures treated with insulin at nanomolar concentrations increased gene and protein expression of tendon markers.²⁹ A single treatment for 24 hours was used and emphasized for clinical relevancy, as treatment could be given concurrently during the time

of surgery. The current work further explored the use of insulin for tendon applications with MSCs grown on biomimetic electrospun micro-nanostructured scaffolds. In a clinical setting, matrices can act as delivery vehicles for cells and biochemical factors. Electrospun fiber matrices are popular and suitable scaffolds for tendon applications, due to their biomimicry to fibers found in natural extracellular matrix (ECM), high surface to volume ratio, and ease of fabrication.³⁰

Both synthetic and natural polymers have been explored for tissue engineering scaffolds. Synthetic polymers are favored due to their engineered properties in terms of mechanical strength, degradability based on the molecular weight, and backbone structure. However, in particular, polyesters lack intrinsic functional groups that endow natural polymers with greater biocompatibility. Conversely, natural polymers suffer from lower mechanical strength, variability, and often require special processing before use. A blend platform combines the advantages of both synthetic and natural materials. Polycaprolactone (PCL) is widely used in tissue engineering due to its favorable physicochemical properties and versatility,^{31–33} as well as its Food and Drug Administration (FDA) approval in sutures and drug delivery systems.³⁴ However, PCL is known for its hydrophobic nature, which can result in decreased cell-material interaction. A blend of PCL with a hydrophilic natural polymer such as cellulose acetate (CA) improved its hydrophilicity, matrix erosion profile, and biocompatibility.^{35,36} In recent years, the global market for CA has been rapidly increasing with a predicted value of approximately 7 billion dollars³⁷ by the year 2021. The relative abundance and flexibility has fueled interest in CA in a myriad of areas including biomedical applications such as tissue engineering and drug delivery.^{38,39}

The present study aimed to further elucidate the effect of insulin supplemented media on the proliferation and differentiation of MSCs into tenocytes in an effort to identify an appropriate dose. Furthermore, this study is the first to report on insulin immobilized PCL-CA electrospun fiber matrices for MSCs tenogenic differentiation on a 3D matrix.

2 | MATERIALS AND METHODS

2.1 | Fabrication of electrospun matrices

PCL (Sigma, Mw = 80 000) was dissolved in dichloromethane:ethanol (85:15) solution at 12 wt% in a 20-mL glass vial. Optimized electrospinning parameters include a flow rate of 2 mL/h, with voltage set at 20 mV at a working distance of 20 cm, in an effort to obtain bead-free fiber matrices. Fiber matrices were collected on a grounded plate with a 10 × 10 cm aluminum foil. Similarly, PCL and CA (Sigma, Mw = 30 000) at various ratios were blended to obtain a 12 wt% solution in trifluoroethanol (TFE) in 20-mL glass vial. The ratio of PCL to CA varied from 25%, 50%, and 75% CA to render four scaffold groups, herein referred to as PCL, 25%CA, 50%CA, and 75%CA. The electrospinning parameters were kept constant at 2 mL/h, and voltage was set at 20 mV at a working distance of 20 cm. Scaffolds were dried and stored under vacuum until use. All scaffolds were immersed in 70% ethanol and exposed to UV on each side for 30 minutes prior to use for cell studies, as previously described.⁴⁰

2.2 | Cell studies

Human MSCs (Millipore) were expanded and used at passage 5. Prior to the start of experiment, cells were serum starved for 24 hours to ensure any possible conflicting or inhibitory growth factors present in the serum were eliminated. These cells were seeded on tissue culture plates, PCL scaffolds, or insulin conjugated PCL-CA blend fibers at a seeding density of 50 000/scaffold on 48-well plate. Media was changed every 2 to 3 days.

For cells studies involving scaffolds, scaffolds were cut into 1-cm² squares and were sterilized with immersion in 70% ethanol and exposure to UV on each side for 30 minutes, as previously described.⁴⁰ After sterilization, scaffolds were placed in 48-well plate dishes and incubated with basal media overnight. At collection, scaffolds were washed with phosphate-buffered saline (PBS) and prepared for analysis.

2.3 | Effect on proliferation

To determine the effect of insulin supplemented media on proliferation, cells were seeded on PCL scaffolds with an initial cell density of 50 000 cells/scaffold. Cells were treated with control (basal) media, or insulin supplemented media containing 1-, 10-, 50-, 100-, or 200-ng/mL concentration. At 6, 12, 24 hours, and at 3, 7, and 14 days, samples were washed with PBS and treated with 1% TritonX and kept at -20°C until all samples were collected. Samples underwent three freeze-thaw cycles to further lyse cells before analysis. A volume of 100 µL of lysate was used to carry out Quant-iT PicoGreen dsDNA Assay Kit.

Fluorescence was measured using a microplate reader, and samples were compared with a standard curve to obtain DNA concentration. Readings from cell-free scaffolds served as scaffold negative controls and were subtracted from sample readings. A sample size of n = 4 was considered for each type of treatment at each time point.

The effect at higher insulin concentrations on metabolic activity of cells was measured on the MSCs cultured in 96 well plates with an initial seeding density of 10 000 cells/well. After 24 hours, cells were incubated with MTS reagent for 2 hours, and absorbance was read at 410 nm using a microplate reader.⁴¹ A sample size of n = 4 for each group was used for these studies.

Scaffolds immobilized with insulin were seeded with 30 000 cells/scaffold (n = 4), and their proliferation rate was measured at days 3, 7, and 14 and treated as previously described.

2.4 | RNA expression of tendon associated genes

For dose response studies MSCs were seeded at 100 000 cells/well in 48-well plates and at day 7 and day 14, RNA was extracted from samples using Trizol reagent. Chloroform was added to Trizol sample solution and centrifuged to isolate RNA lysate layer. The RNA was collected and washed with isopropanol and 80% ethanol. Total RNA was quantified using a nanodrop (Thermoscientific) instrument. A total of 1 µg of RNA was used for cDNA synthesis (cDNA synthesis; Biorad). Real-time polymerase chain reaction (RT-PCR) was conducted using iTaq SYBRgreen kit (Biorad) on Biorad CFX96 machine. Collagen I (col I), collagen III (col III), scleraxis (scx), tenomodulin (tnmd), and decorin (dcn) were

evaluated and normalized to housekeeping gene, β -actin and 18s. Primer sequences used for PCR are listed in Table S1.

Tenogenic gene expression in comparison with control growth factor IGF-1 were performed on MSCs seeded on electrospun nanofiber matrices and cultured in media supplemented with either IGF-1 or insulin. In brief, fiber matrices were seeded 100 000 cells/scaffold and were treated with basal media, 100-ng/mL insulin supplemented media, or 250-ng/mL IGF-1 supplemented media. Samples (n = 4) were collected at days 3, 7, 14, and 21 and treated as previously described.

Similarly, insulin immobilized scaffolds were seeded with 100 000 cells/scaffold and treated with basal media. At various time points of days 3, 7, and 14 samples (n = 4) were collected. RNA was extracted and qualitative PCR analysis was conducted as previously described.

2.5 | Dimethylmethylene blue assay

Sulfated glycosaminoglycans (S-GAG) was quantified using dimethylmethylene blue (DMMB) assay. Samples (n = 4) were washed and digested with proteinase K overnight in 56°C. A 50- μ L cell lysate was reacted with 200- μ L DMMB solution, and absorbance was read at 520 nm using a plate reader. A standard curve was used to quantify S-GAG as per the reported methods.⁴² Readings from cell-free scaffolds (negative controls) were subtracted from sample readings.

2.6 | Immunofluorescence

For immunofluorescence, scaffolds were washed with PBS and fixed with formalin for 15 minutes at room temperature. Scaffolds were washed with cold PBS for 5 minutes, twice. Scaffolds were permeabilized with 0.25% TritonX100 solution and incubated for 10 minutes. Triton was removed, and scaffolds were again washed with cold PBS for 5 minutes. The scaffolds were blocked with 3% BSA for 30 minutes at room temperature. The BSA solution was then removed, and primary antibody for collagen I (rabbit polyclonal; Abcam) was added in 1% BSA solution for 1 hour. The primary antibody was removed, and the scaffold was washed thrice in PBS for 5 minutes. Secondary antibody was then added for 40 minutes, followed by subsequent PBS washing step, for 5 minutes, thrice. Dapi staining was added prior to viewing.

2.7 | Scaffold characterization

2.7.1 | Fiber diameter—Scaffolds were sputter coated with gold/palladium and viewed under SEM at 500 $\times$ magnification. Using Image J software, 100 fibers from three images at three different locations were measured to give an average fiber diameter for each polymer composition.

2.7.2 | Contact angle—Contact angle was measured using a goniometer (rame-hart Model 100). A drop of 5 μ L of water was placed on 1-cm² scaffold, and the resulting contact angle was recorded immediately thereafter. A total of six readings were averaged to obtain the average contact angle for each polymer composition.

2.7.3 | Tensile testing—Tensile mechanical testing was conducted using 10 mm× 20 mm sheets, per ASTM standards on an Instron Tensile machine using a 50-N load cell.⁴¹ Scaffolds were incubated in PBS overnight prior to testing. A total of six measurements were averaged to obtain the tensile parameters for each polymer composition.

2.8 | Cellular attachment on blend scaffolds

Human MSCs, in passage 5, were seeded on the scaffolds, with an initial seeding density of 50 000 cells. After 24 hours, scaffolds were washed with PBS and collected in 2-mL tubes treated with 1% TritonX and kept at −20°C until all samples were collected. Samples underwent three freeze-thaw cycles to further lyse cells before analysis. A volume of 100 µL of lysate was used to carry out Quant-iT PicoGreen dsDNA Assay Kit. Fluorescence was measured using a microplate reader, and samples were compared with a standard curve to obtain DNA concentration. Readings from cell-free scaffolds served as scaffold negative controls and were subtracted from sample readings. A sample size of $n = 4$ was considered for each type of treatment at each time point.

2.9 | Insulin delivery

Insulin was attached to carboxylic moieties of the polymer backbone using a standard carbodiimide chemistry as per manufacturer's instructions.⁴³ After sterilization, 1-cm² scaffolds were placed in 24-well plate dishes and treated with 500mM of sterile NaOH for 1 hour to cleave ester bonds present in PCL. This allowed for free carboxyl groups on the scaffolds to partake in conjugation. The scaffolds were then incubated in sterile 100mM MES buffer for 1 hour. The MES buffer was removed, and fresh MES buffer containing coupling reagents EDC/NHS (500 µg/mL) and insulin (10 µg/mL) were added to the wells. The well plates were wrapped with parafilm and placed on a rocker overnight. After conjugation, the MES conjugation buffer was removed, and the scaffolds were washed with sterile water repeatedly five times to remove unreacted products.

2.10 | Quantification of insulin

Insulin was measured using microBCA assay directly on the insulin immobilized scaffolds as per earlier reports published elsewhere.^{43,44} Scaffolds with and without crosslinking agents were measured to see the amount of insulin adsorbed onto the scaffolds. The amount of insulin adsorbed was subtracted from the total insulin content to obtain the amount of immobilized insulin. For all these measurements, a sample size of $n = 5$ was used.

2.11 | Statistical analysis

All data are expressed as means ± standard deviation (SD) for at least triplicate number of samples. Statistical analysis was carried out using one-way ANOVA and Tukey's post hoc analysis for multiple comparisons to determine significance using Graph Pad Prism software. A value of $p < 0.05$ was considered significant.

3 | RESULTS AND DISCUSSION

3.1 | Proliferative effect of insulin doses

Insulin effect on proliferation was measured using DNA concentration collected from scaffolds at 6, 12, and 24 hours and 3, 7, and 14 days following cell seeding. All groups had slight increase in DNA content over time despite the low serum condition (Figure 1A). In our preliminary studies, we found a 2% fetal bovine serum (FBS) concentration was able to maintain cell populations without dramatic increase or decrease in cell population (Figure S1). No significant differences were found in cell proliferation among cells treated with or without insulin supplemented media on fiber scaffolds at concentrations up to 200-ng/mL insulin. The data suggests, in the presence of low serum conditions, within the range of concentrations tested, insulin did not have an effect on the proliferation rate. Mitotic effects of insulin appear to be highly dependent on cell type and concentration.^{7,9,24} For instance, human lung fibroblast cells showed significant increase in proliferation after 24 hours post treatment with 2- μ g/mL insulin.²⁴ Similarly, human hepatic stellate cells treated with approximately 6-ng/mL insulin showed significant increase in proliferation.⁷ Insulin treatment on human umbilical cord matrix derived MSCs showed no effect on proliferation at insulin concentrations below approximately 6 μ g/mL but significant increase in proliferation with concentrations above 14.5 μ g/mL.⁹

To determine if higher insulin concentrations was needed to induce proliferative effect on human bone marrow derived MSCs, metabolic activity of cells grown on tissue culture plate was measured after 24 hours of treatment. Our findings showed significant increase in metabolic activity with cells treated with concentrations of 1- μ g/mL insulin or higher (Figure 1B). This corroborates with the findings of Li et al who observed increased proliferation in umbilical cord matrix derived MSCs at concentrations above 6 μ g/mL. The concentration of insulin needed to stimulate proliferation may depend on the number of insulin receptors present on the cell membrane. A lower number of insulin receptors present in the cell may require higher doses of insulin to elicit a proliferative effect.⁹ No studies to our knowledge have been conducted on the abundance of insulin or IGF-1 receptors on human MSCs. Higher microgram insulin concentrations favor not only progenitor proliferation but also chondrogenic differentiation.^{9,26,45–47} Therefore, only lower insulin concentration ranges were used to characterize dose responses.

3.2 | Gene expression of dose studies

Insulin's potential as a differentiation factor has been widely explored for cartilage applications,^{25,27,28,48,49} with only one known publication in the area of tendon regeneration, as previously described.²⁹ However, insulin's analogue IGF-1 has been widely researched for tendon regeneration^{8,10,16} and shown to increase collagen production,⁷ the main constituent of tendon ECM. For this reason, insulin may have potential in tendon tissue engineering. Dose studies with cells seeded on tissue culture plates showed higher expression of tendon markers with cells treated with insulin supplemented media when compared with the control group (Figure 2). Scleraxis, a transcription factor that is highly expressed by tendon progenitor cells,^{50,51} was significantly upregulated at day 7 for cells treated with insulin concentrations equal to and above 10-ng/mL insulin. A dose-dependent

expression of scleraxis was found at day 14, with 100- ($p = 0.01$) and 200-ng/mL ($p = 0.001$) insulin-treated groups maintaining significant upregulation. Expression of collagen I was also dose dependent, and significant increase was observed with cells treated with 100-ng/mL insulin and 200-ng/mL concentrations at day 7 ($p = 0.05$) and day 14 ($p = 0.001$). No dose-dependent pattern was found in the expression of ECM markers, collagen III, and decorin. Tenomodulin, a mature marker for tendon,⁵² was found to be significantly increased at day 14 with cells treated with 200-ng/mL insulin ($p = 0.01$). From the data, it was gathered the minimum effective dose for increase in overall tenogenic markers was 100 ng/mL. Although tenomodulin was only upregulated with 200-ng/mL concentration of insulin, a concentration of 100 ng/mL induced upregulation of collagen I and scleraxis. The lower concentration was preferred due the potential of cartilage differentiation at higher concentrations and possible interaction of exogenous insulin on glucose homeostasis. Thus, 100 ng/mL of insulin concentration was chosen to move forward in testing with electrospun fibers.

3.3 | Comparison with IGF-1

IGF-1 has been shown to have versatile roles in tissue engineering including mitotic and anabolic effects.^{8,10,16} Protein insulin is structurally analogous to IGF-1¹⁹ and has been shown to bind to IGF-1 receptors to elicit similar effects^{9,20,26,29} yet is more widely available at lower costs than IGF-1. An insulin group treated with 100 ng/mL of insulin supplemented media was compared with cells treated with 250-ng/mL IGF-1 supplemented media on electrospun PCL scaffolds. The IGF-1 dose was taken from literature as optimal for low serum conditions, as described by Herchenhan et al.⁸ When hMSCs were seeded on PCL electrospun fibers and treated with insulin or IGF-1 supplemented media, there was a slight increase in scleraxis, but none were found to be significant. Expression of tenomodulin was upregulated at day 7 ($p = 0.001$) (Figure 3). This observed upregulation in tenomodulin, a mature tendon marker, at day 7 suggests the possibility that expression of scleraxis may have peaked prior to the first time point at day 3 and that cells may be progressing at a faster rate. Significant upregulation of collagen I expression was achieved with cells treated with both insulin and IGF supplemented media, with no significant difference between the two groups. This corroborates with past publications that have observed increased collagen expression with insulin²⁴ or IGF-1 treatment.^{8,10} When measuring for total sulfated GAG content on the fiber scaffolds, significantly more GAG was found on scaffolds in the treated groups at day 21 (Figure 4). There was no difference between the insulin and IGF group in the amount of GAG produced. Though GAGs make up a small percent of native tendon tissue, their presence in tendon ECM serve to protect tendons against shear and compressive forces.⁵³ Additionally, GAGs have been shown to play a role in collagen fibrillogenesis, fiber diameter, and mechanical integrity.⁵⁴ Furthermore, increased expression of collagen I protein was observed to be qualitatively increased with cells grown on electrospun fibers after treatment with insulin or IGF-1 when compared with the control basal group (Figure 4).

The data supports expression of tendon markers with insulin supplemented media at the same levels as IGF-1 supplemented media; thus, insulin may be a feasible biomolecule alternative for tendon differentiation. Furthermore, due to the wide availability of insulin, it

is feasible that insulin could be a component of a standardized tendon differentiation media. Further tests need to be conducted to understand the mechanism behind insulin effects. It remains unknown whether insulin is binding to the IGF-1 receptors and mediating the IGF-1 receptor pathway or if insulin is binding to its own receptor. Further complexity arises with the consideration that IGF-1 and insulin receptors can form hybrids. Insulin was observed to selectively bind to insulin receptors at physiological concentrations in endothelial cells, but at concentrations above the physiological dose, shown to bind to IGF-1 receptors.²⁰ The affinity of insulin to IGF-1 receptors may vary between cell types; thus, further analysis on MSCs need to be conducted to better understand the mechanism of insulin on tendon differentiation.

3.4 | Fabrication and characterization of blend scaffolds

A PCL-CA blend was chosen to combine the advantages of both synthetic and natural polymers. PCL is a well-characterized polyester for tissue engineering and drug delivery applications^{34,55–57} due to its controllable properties. Various methods such as surface modification and formation of PCL composites can result in alterations in the polymer's mechanical properties, degradation, and bioactivity.^{57,58} However, one main advantage of natural polymers over synthetic polymers is their innate biological motifs that contribute to overall better bioactivity and biocompatibility. The presence of biologically functional moieties allows for greater interactions with cells and proteins.^{59,60} A blend of PCL with CA, a natural hydrophilic polymer, was chosen to improve matrix hydrophilicity, biocompatibility, and promote tissue regeneration.⁶⁰

At the optimized conditions SEM images of PCL-CA blend scaffolds showed bead-free, continuous fibers (Figure 5A). Properties of scaffolds are listed in Table 1. With increasing CA concentrations, there was a decrease in fiber diameter (Figure 5B). A similar trend was found with PCL-CA blend fibers fabricated by Ahmed et al.⁶¹ The addition of CA also lowered the tensile strength of the scaffolds, with significantly less maximum load associated with the blend fibers due to its hydrophilic and fibrous nature in the when electrospun (Figure 5C). Similarly Ding et al fabricated electrospun PVA-CA blend scaffolds and found increasing CA content in the blend scaffolds decreased overall mechanical strength.⁶²

With increasing CA concentrations, the water contact angle was decreased (Figure 5D). This was expected as PCL is highly hydrophobic, whereas CA is hydrophilic. DNA concentrations from seeded cells were found to be higher on the blend scaffolds when compared with PCL only group (Figure 5E) after 24 hours, indicating greater cell attachment. Significantly, higher DNA content was found on the 25%CA group when compared with PCL ($p < 0.05$). This is attributed to the decrease in hydrophobicity of the scaffold, which would have allowed for greater cellular attachment. However, it was observed with scaffolds containing greater percentage of CA, there was a decreasing trend in the amount of cellular attachment despite decreasing hydrophobicity. Though several studies support improved cell attachment on hydrophilic versus hydrophobic surfaces,^{63,64} other studies reported enhanced cell attachment at intermediate levels of hydrophobicity and hydrophilicity.^{65,66} Cell adhesion on extremely hydrophobic or hydrophilic surface were

decreased. This may be explained through studies that show enhanced protein adsorption on hydrophobic surfaces. Initial cell attachment on biomaterials is in part aided by initial adsorption of adhesion proteins to the material⁶⁷; thus, a balance between hydrophobicity and hydrophilicity that promotes both protein adhesion and cellular-material interactions may be more favorable for cell attachment. Fiber diameter may also be playing a role in cellular attachment. On PCL electrospun fibers, Peach et al found increased attachment of MSCs on scaffolds with average fiber diameters of 900 to 1000 nm, when compared with scaffolds with fiber diameters in the 400 to 500 nm range.⁶⁸ Increasing CA concentrations decreased the average fiber diameter of the scaffolds, which may have resulted in lower porosity and subsequently lower cell infiltration⁴¹ during the first 24 hours.

There are several methods that can be employed to deliver biomolecules on electrospun fibers including physical adsorption, coelectrospinning, and chemical conjugation. Physical adsorption, the simplest method, relies on relatively weaker interactions such as electrostatic forces, hydrogen bonding, and van der Waals interactions that can be difficult to control.⁶⁹ Biomolecules that are added to the polymer solution for coelectrospinning run the risks of degradation from solvents used in the solution.⁴³ Chemical conjugation is a much milder process that is conducted in aqueous solutions, which helps preserve the bioactivity of the biomolecule. Additionally, chemical conjugation of protein on the surface of scaffolds allows for greater control and longer activity of the immobilized biomolecule.⁶⁹ Cells can directly interact with the immobilized insulin and modulate cellular behavior. Thus, chemical conjugation was implemented to functionalize the scaffolds with insulin.

The electrospun fibers were initially hydrolyzed to provide ester groups as sites for conjugation of insulin to the fibers. No significant difference was found in the amount of immobilized insulin between PCL and 25%CA scaffolds, which measured 67 and 76 ng of insulin per 1 cm² area, respectively. However, significantly lesser amounts of immobilized insulin were measured on 50%CA and 75%CA scaffolds, 26 and 8 ng, respectively. The amount of immobilized insulin on the PCL and 25%CA scaffolds are in range with other similar reactions on PCL fibers. Cho et al observed 49 ng of neural growth factor conjugated to PCL/PEG electrospun fibers,⁷⁰ whereas Cheng et al. found 120 ng of collagen immobilized on PCL/chitosan fibers.⁴³ Based on the findings with regards to tensile strength, cellular attachment, and amount of immobilized insulin, the scaffold composition with 25% CA was chosen to conduct biological feasibility studies.

3.5 | Bioactivity of insulin functionalized scaffolds

Clinically, insulin delivery must be dealt with cautiously as insulin is an important and integral part of normal glucose homeostasis. The concentrations tested in these studies are far below the international unit of insulin (34.7 µg/IU) and thus are not expected to have systemic effects. Moreover, insulin was immobilized onto the scaffold, thus reducing a possible burst release effect. Insulin released into the media was measured during the duration of the study. By day 14, roughly 19% of the insulin was eluted into the media (Figure S2). This demonstrates a slow release of insulin and demonstrates the avoidance of a burst release effect commonly associated with biomolecules that are physically encapsulated.

At day 14, there was significant decrease in DNA content with the insulin conjugated scaffolds when compared with the control group ($p = 0.001$) (Figure 6A). This may indicate that cells seeded on the insulin conjugated scaffolds are expending energy towards differentiation rather than proliferation, as evidence indicates an inverse relationship between the two cellular modalities.⁷¹ Earlier findings showed no difference in proliferation with cells on PCL scaffolds with insulin supplemented media. This may be due to experimental differences. For insulin immobilized scaffolds, a lower initial seeding density was used to ensure cells adequate space to proliferate. This resulted in overall less DNA content but a visible and significant increase in DNA concentration at day 14 for cells treated on control scaffolds.

In terms of differentiation markers, there was an upregulation of scleraxis observed at day 7 ($p = 0.05$) with a significant increase in tenomodulin at day 14 ($p = 0.05$) (Figure 6B). When compared with cells treated directly with insulin supplemented media, cells grown on insulin immobilized scaffolds seem to have a slower progression as evidenced by later increase in tenomodulin at day 14, rather than day 7 as was seen with the former. This delay in tenomodulin expression from cells grown on insulin functionalized scaffolds, when compared with cells treated with insulin supplemented media, may be due the accessibility of cells to the insulin protein. Chemical conjugation of biomolecules onto the surface of fibrous scaffolds may potentially decrease bioactivity. Due to chemical modification, cells may not recognize the biological ligands of the biomolecule, as they may not be fully exposed.⁶⁹ It is important to note however, that cells grown on the insulin immobilized scaffolds still had an upregulation of tendon markers. This suggests the bioactivity of the immobilized insulin on the scaffolds was preserved and remained bioactive. However, increased cell density and growth of ECM on the scaffold may potentially block access to the immobilized insulin at later time points.

The influence of growth factors on tissue development and healing make them attractive biomolecules to stimulate and differentiate cells towards desired lineages. IGF-1 is widely researched in musculoskeletal tissue engineering across all tissues due to its mitotic and growth promoting effects. Specifically, for tendon applications, IGF-1 has been shown to promote collagen synthesis^{10,12,16} when delivered directly to the tendon tissue. However, direct administration of the growth factor as a single dose may not allow for long-term effects.¹² Covalent conjugation can extend the half-life and provide continuous exposure to the biomolecule.⁷² Additionally, limitations associated with growth factors have prompted researchers to look for growth factor alternatives, such as small molecules, peptides, and other proteins. The analogous structures of insulin and IGF-1, as well the observed overlapping effects of insulin, makes insulin an attractive candidate as a substitute for IGF-1.

4 | CONCLUSION

Regulatory hurdles, along with other limitations such as short half-life and high costs, have fueled research towards growth factor alternatives, such as small molecules and other proteins. The biomolecule insulin was explored for potential use in tendon tissue engineering. Mesenchymal stem cells treated with insulin supplemented media showed increased expression of tendon-related markers and, when compared with growth factor

IGF-1, showed similar levels of expression. This indicates a possibility for the use of insulin as a bioactive component in the creation of a standardized tendon differentiation media. Additionally, electrospun scaffolds of PCL and CA blends were characterized for their potential as clinically relevant insulin delivery systems. Functionalized scaffolds with immobilized insulin showed similar effects on MSCs as cells treated with insulin supplemented media, indicating the preservation of insulin bioactivity. The data suggest potential for the use of insulin bioactive scaffolds for tendon repair and regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

Funding support for this work was provided by the National Institute of Biomedical Imaging and Bioengineering of the National Institutes of Health (Award Number R01EB020640), and the Connecticut Regenerative Medicine Research Fund (15-RMB-UCHC-08).

Funding information Connecticut Regenerative Medicine Research Fund, Grant/Award Number: 15-RMB-UCHC-08; National Institute of Biomedical Imaging and Bioengineering, Grant/Award Number: R01EB020640

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FIGURE 1.

Effect of cells treated with various doses of insulin supplemented media in low serum conditions on electrospun fibers. A, Proliferation—DNA concentration of all groups indicate increased DNA content over time but no significant difference between control and insulin treated groups. B, At higher concentration of insulin in serum free media shows higher metabolic activity with cells treated with 1 µg/mL or higher insulin after 24 hours

FIGURE 2.

Dose dependent study on expression of tendon-related markers. Statistically significant increase in expression of scleraxis, collagen I, collagen III, and decorin with cells treated with 100- or 200-ng/mL insulin supplemented media. (*statistically significant from control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; #statistically significant between groups: # $p < 0.05$ ## $p < 0.01$, ### $p < 0.001$)

FIGURE 3.

Expression of tendon related markers with cells treated with 100 ng/mL of insulin or 250-ng/mL insulin-like growth factor-1 (IGF-1) supplemented media. No significant differences between insulin and IGF-treated groups in the expression of scleraxis, collagen I, and decorin for all time points. (#statistically significant between groups: # $p < 0.05$; *statistically significant from control group: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

FIGURE 4.

Extracellular matrix (ECM) protein analysis of cells treated with insulin-like growth factor-1 (IGF-1) or insulin. A, Cells treated with IGF-1 or insulin supplemented media showed significantly higher GAG content when compared with cells treated with basal (control) media. B, Increased expression of collagen I was qualitatively observed from cells treated with IGF-1 or insulin when compared with basal group [Colour figure can be viewed at wileyonlinelibrary.com]

FIGURE 5.

A, SEM image of fabricated scaffolds. B, Fiber distribution based on SEM images. C, Maximum load. D, Water contact angle. E, Cellular attachment. F, Insulin loading on scaffold groups

FIGURE 6.

A, DNA concentration from cells grown on insulin conjugated fibers. B, Expression of tendon markers with cells grown on insulin immobilized fibers show similar trends to cells treated with insulin supplemented media

TABLE 1

Physicochemical properties of micro-nanostructured fiber matrices

Scaffold Composition	Fiber Diameter, nm	Max Load, N	Water Contact Angle	Cellular Attachment, ng/mL	Immobilized Insulin, ng
PCL	732 ± 335	10.79 ± 1.49	116 ± 9.19	22.76 ± 15.46	67.00 ± 24.08
25%CA	886 ± 426	6.53 ± .48	114 ± 5.65	95.88 ± 7.76	76.78 ± 18.97
50%CA	724 ± 350	5.33 ± .82	106 ± 2.12	64.79 ± 27.54	26.66 ± 5.46
75%CA	474 ± 305	2.90 ± 0.42	105.5 ± 0.7	54.65 ± 16.86	8.47 ± 7.0