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Osteoblast biocompatibility of pre-mineralized, hexamethylene-1,6-diaminocarboxysulphonate crosslinked chitosan fibers

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Abstract

Biopolymer-ceramic composites are thought to be particularly promising materials for bone tissue engineering as they more closely mimic natural bone. Here, we demonstrate the fabrication by electrospinning of fibrous chitosan-hydroxyapatite composite scaffolds with low (1 wt%) and high (10 wt%) mineral contents. Scanning electron microscopy (FESEM), energy dispersive spectroscopy (EDS) and unidirectional tensile testing were performed to determine fiber surface morphology, elemental composition, and tensile Young's modulus (E) and ultimate tensile strength (σ_{UTS}), respectively. EDS scans of the scaffolds indicated that the fibers, crosslinked with either hexamethylene-1,6-diaminocarboxysulfonate (HDACS) or genipin, have a crystalline hydroxyapatite mineral content at 10 wt% additive. Moreover, FESEM micrographs showed that all electrospun fibers have diameters (122 – 249 nm), which fall within the range of those of fibrous collagen found in the extracellular matrix of bone. Young's modulus and ultimate tensile strength of the various crosslinked composite compositions were in the range of 116 – 329 MPa and 2 – 15 MPa, respectively. Osteocytes seeded onto the mineralized fibers were able to demonstrate good biocompatibility enhancing the potential use for this material in future bone tissue engineering applications.

Keywords

chitosan; hydroxyapatite; genipin; diisocyanate; composite; electrospinning; bone scaffold

1. Introduction

Bone injuries and damage are a serious economic and health concern worldwide. In a 2011 report of the American Academy of Orthopaedic Surgeons, bone-related disorders were the

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leading cause of disability, amounting to a total national expenditure (*i.e.* health care costs (direct) and lost wages (indirect)) of \$950 billion which is equivalent to 7.4% of the national gross domestic product of the US during 2004–2006 [1]. Common bone fracture treatment options are often limited either by the availability of materials that can be used for autografting or by the success rate of current allografting techniques [2]. This leads to a continuously increasing demand for bone substitute materials for repair, regeneration or replacement and currently drives the growing research interest in the design and fabrication of materials that mimic the natural structure, composition and properties of bone.

As a composite material, natural bone typically consists of 6 – 13% water, 49 – 70% mineral, 24 – 38% inorganic phase and the remainder organic materials [3]. The inorganic matter primarily consists of hydroxyapatite $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$ crystals, which are embedded in an organic matrix that is mainly composed of type-1 collagen [4]. Current research efforts primarily focus on synthetic porous hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$) as resorbable bone substitute material due to its biocompatibility and chemical composition [5]. Additionally, bone tissue scaffolds frequently also contain biocompatible polymers to emulate bone-like chemical and mechanical properties [6]. Historically, synthetic polymers [7] such as polyethylene oxide (PEO) [8], polymethylmethacrylate [9], polyethylene terephthalate [10], poly- ϵ -caprolactone (PCL) [11] and polylactic-*co*-glycolic acid [12] have been of interest, although collagen [13], alginate [14], soy/gelatin [15], fibrin [16], cellulose-based [17], chitin [18] and chitosan [19] biopolymers are now gaining more attention due to their versatile chemistry, resorbability, bioactivity, water-uptake ability, improved biocompatibility and non-toxicity.

Chitosan, the linear and partially acetylated (β -(1-4)-2-amino-2-deoxy-*D*-glucan) biopolymer, is used as the polymer matrix for biomimetic bone applications because it is inexpensive, abundant, and easy-to-process in comparison to other biopolymers [20, 21]. Previously demonstrated processing techniques for chitosan polymer blends as materials for bone tissue scaffold include freeze-casting [22], freeze-drying [23] and electrospinning [24–28]. Electrospinning is an inexpensive, scalable and flexible technique that creates fibrous and porous scaffolds with microstructures that mimic the extracellular matrix of the cell [29]. Most of the work on chitosan-based electrospun scaffolds for bone regeneration has used blends of synthetic polymers, such as PEO [25, 26] or polyvinyl alcohol (PVA) [24], to facilitate fiber formation. Although the blending of synthetic with natural polymers can be beneficial in facilitating biopolymer fiber formation, the downside can be a delayed cellular response and onset of growth during cell culture, due to the presence of the additional synthetic polymer in the scaffold [27].

Biopolymeric materials are often sensitive to high moisture environments; therefore most scaffolds are crosslinked to improve the chemical or mechanical stability of the resulting material. Recently, we reported the novel crosslinking of electrospun chitosan fibers with the crosslinkers genipin and hexamethylene-1,6-diaminocarboxysulfonate (HDACS) [30]. Genipin is a natural crosslinker that has demonstrated success in a variety of scaffolds and tissue cultures including bone tissue engineering applications [34–37]. Both crosslinkers have been previously utilized for chitosan gels and films [31]. Moreover, both have been favored over the more commonly used glutaraldehyde, due to their lower cytotoxicity,

ability to react under a wide range of pH and temperature, and to improve the chemical and mechanical properties of the scaffolds [32].

This work describes the development of a biomimetic bone scaffold using porous electrospun crosslinked chitosan-hydroxyapatite composite fibers, which will potentially be favorable for bone cell attachment, proliferation and tissue regeneration. More specifically, two unique approaches are introduced: 1) a one-step addition of both the porous hydroxyapatite (nHAp) and the crosslinker to the spinning solution and 2) the fabrication of an electrospun porous hydroxyapatite-chitosan composite that is, in a second step, crosslinked with either HDACS or genipin. The preosteocyte cell line, MLO-A5, was used to evaluate the potential of these mineralized fibrous scaffolds for bone tissue engineering applications.

2. Method

2.1 Materials and preparation of chitosan-nHAp solution

All methods used for the preparation of solutions for electrospinning and crosslinking were based on those described by Austero *et al.* [30]. Here, 3.7 wt% chitosan (75–77% degree of deacetylation (DD), $M_w = 190 - 310$ kDa, Sigma Aldrich, St. Louis, MO) in trifluoroacetic acid (TFA, 99% ReagentPlus, Sigma Aldrich) was mixed (24 h, $23 \pm 2^\circ\text{C}$). Prior to electrospinning, 1 or 10 wt% of hydroxyapatite nanopowder (nHAp, <200 nm particle size BET 97%, surface area >9.4 m²/g, Sigma Aldrich) was sonicated with the chitosan solutions for approximately 2 min. HDACS was prepared using the protocol reported by Welsh *et al.* [33], while genipin was used as received (Wako Pure Chemicals Industry, Ltd., Japan). The crosslinkers were added as previously reported by Austero *et al.* 2012; either crosslinker was mixed for approximately 2 min prior to loading of the solution into the syringe for electrospinning.

2.2 Electrospinning of chitosan-nHAp solution

Electrospinning was carried out at $23 - 25^\circ\text{C}$ and 30 – 35% relative humidity using a syringe-collector distance of 10 cm and an applied voltage of 15 kV. The solution was electrospun at a flow rate of 0.5 mL/h. The mats ($n = 3$ per composition) were collected on a 90 mm $\times$ 90 mm aluminum-foil wrapped copper plate. Post-treatment at 120°C for 2 h [30] was performed for the HDACS to activate the crosslinking reaction. Uncrosslinked mats with 1 and 10 wt% nHAp contents were spun as controls. The mat morphology was compared to that of pure chitosan mats of an earlier study [30].

2.3 Characterization of electrospun chitosan-nHAp composite fibers

The mat surface morphology and elemental analysis were performed using a Zeiss Supra 50VP FESEM (Carl Zeiss NTS, LLC) equipped with and an EDS system (Oxford Instruments, Oxford, UK) on samples that had been sputter coated with Pt/Pd at 40 mA for 35 s (Cressington Scientific Instruments, Watford, UK). Fiber diameters ($n = 150$ or three independent mats $\times$ 50 fibers per mat) were measured using ImageJ (version 1.41o, NIH). Statistical significance was evaluated in StatPlus:Mac LE2009 (Build 5.8 AnalystSoft, Inc.); a p -value of less than 0.05 was considered statistically significant.

The Young's modulus (E) and ultimate tensile strength (σ_{UTS}) of the spun mats were determined from tests on an Instron 5500R (Model 1125, Instron, Norwood, MA). For each mat composition ($n = 3$ mats/composition), three $10 \text{ mm} \times 35 \text{ mm}$ strips were cut from each of the three mats ($n = 9$ strips/composition). Thickness (mean + standard deviation) values for the tested mats can be found in Supplemental Image 1. For tensile testing, the strips were fixed with double-sided tape between two square cardboard frames having an opening of $25 \text{ mm} \times 25 \text{ mm}$ to protect them from loading before testing. The frames were held with pneumatic grips and their vertical sides were manually cut with scissors immediately before testing. Tensile tests were performed with a 500 g load cell at a strain rate of 0.02 s^{-1} at 21°C and 65% relative humidity (RH). The force-displacement data were corrected for slack and converted into engineering stress and strain using the mat's initial effective cross-sectional area, calculated from the area density of the samples according to the previously reported method [32]; the frame opening of 25 mm was used as the gauge length. The modulus of the mat is the initial linear slope of the stress-strain curve, the maximum stress, which the mat could support, is its tensile strength (σ_{UTS}).

2.4 Culture of MLO-A5 preosteocyte cells

MLO-A5 cells were generously donated by the Bonewald Laboratory (University of Missouri-Kansas City) and were cultured in α -MEM containing nucleotides and L-glutamine (Life Technologies, Grand Island, NY) [38]. Media was supplemented with 5% fetal bovine calf serum (FCS) and 5% supplemented bovine calf serum (BCS) purchased from ThermoScientific Hyclone (Rockford, IL). Cells were plated into 100 mm^2 culture plates (BD Biosciences, San Jose, CA) in a Revco Ultima II tissue culture incubator at 37°C and 5% CO_2 (ThermoScientific, Ashenville, NC) and split every three days.

2.5 Cellular visualization on electrospun fiber mats

Immunofluorescent (IFC) staining was performed in order to determine the morphology of MLO-A5 cells on all of the chitosan fiber compositions. Prior to cell seeding, mineralized chitosan-fiber mats were prepared and sterilized in a 70% ethanol (EtOH) solution (Pharmco-AAPER, Brookfield, CT) followed by a 1X phosphate-buffered saline (PBS) wash (Corning CellGro). Subsequently, mats were placed into a 6-well cell culture plate (BD Biosciences, San Jose, CA) and allowed to remain under the UV light within the Baker Sterigard laminar cell culture hood overnight (Sanford, Maine). After preparation of each type of mineralized fibrous mat, cells were seeded at a density of 5×10^4 cells/well. Fibronectin coated glass coverslips (BD Biosciences, San Jose, CA) were used as a control. Cell cultures were washed twice with 1X PBS 48 h after growth and stained according to standard protocol. Briefly, MLO-A5 cells were fixed on the various fibrous mats with a 4% paraformaldehyde (PFH) solution (Polysciences Inc, Warrington, PA) in 1X PBS for 30 min and stained with $0.1 \text{ }\mu\text{L/mL}$ DAPI (Invitrogen/Life Technologies, Grand Island, NY) for the nucleus or $5 \text{ }\mu\text{L/mL}$ of Oregon Green 488 phalloidin (Invitrogen/Life Technologies, Grand Island, NY) for the actin cytoskeleton. IFC were taken using a Zeiss confocal microscope (Carl Zeiss LSM 700) and used to visualize the MLO-A5 cells on the various fiber compositions. Images were also color inverted in order to highlight cytoskeletal features that were difficult to detect.

Using FESEM, additional cytoskeletal features present among MLO-A5 cells grown on the various mineralized fiber mats were identified. MLO-A5 cells were fixed with Karnovsky's Fixative (5% glutaraldehyde, 4% formaldehyde, 0.08 M sodium phosphate buffer) (Electron Microscopy Sciences, Hatfield, PA) for 1 h. Afterwards samples were exposed to a serial EtOH dehydration series with each exposure lasting 10 min. Samples from both Day 2 and Day 9 were prepared using this protocol and then immersed in 99.9% EtOH and chemically dried using hexamethyldisilazane (HMDS) (Electron Microscopy Sciences, Hatfield, PA). FE-SEM samples were sputter coated with Pt/Pd at 40 mA for 35 s (Cressington Scientific Inc., Watford, U.K.) and loaded into a Zeiss Supra 50 VP FE-SEM at high vacuum.

2.6 Quantitative analysis of cellular cytoskeletal filopodia

Filopodia, identified by their length and number, were measured from the SEM micrographs with ImageJ according to a previously established protocol [39]. Briefly, each FESEM micrograph at both Day 2 and Day 9 time points were background subtracted with a rolling ball radius of 50 pixels. Scale bars were normalized. Filopodia were measured and counted to create a distribution histogram from at least three representative micrographs.

2.8 Cell viability using Promega Cell Titer Glo Assay

In order to assess viability of cells grown on the various mineralized chitosan fiber mats, the Promega Cell Titer Glo assay (Promega, Madison, WI) was utilized to qualitatively measure the amount of adenosine triphosphate (ATP) in culture. Both chitosan-genipin and chitosan-HDACS mats were cut into squares (about 1.4 cm × 1.4 cm), covering approximately 90% of the 2.0 cm² area of each well. Cells were seeded at a density of 5 × 10⁴ cells/well and grown in 500 µL of the supplemented α-MEM media. The control was MLO-A5 cells seeded at the same density into the tissue culture polystyrene (TCP) wells. Media was changed every three days during this experiment. After each time point, the assay was performed according to the Promega Protocol. Briefly, the luminescent reagent was mixed with the contents of the well and shaken for 2 min to induce cell lysis. After allowing the signal to stabilize for 10 min, the entire contents of the wells were placed into a Turner Biosystems 20/20ⁿ Luminometer (Promega, Madison, WI) and scanned using the preloaded Promega Cell Titer Glo protocol. Measurements for each well were recorded three times, with the reported values being the average with standard deviation.

2.7 Quantitative analysis of osteocyte markers using real-time quantitative PCR (qPCR)

In order to quantitatively measure the genetic levels of alkaline phosphatase (Applied Biosystems, Mm01187115-m1), the Taqman Gene Expression Assays (Applied Biosystems-Life Technologies, Grand Island, NY) were used with β-actin (Applied Biosystems, Mm00607939-s1) as the control housekeeping gene. MLO-A5 cells were seeded onto either the CS-genipin mineralized or CS-HDACS mineralized series at a density of 5 × 10⁴ cells/well and grown in 2 mL of the supplemented α-MEM media. Fiber mats were isolated from culture wells on Day 2, 3, and 4. No additional time points were considered necessary because of prior results obtained on non-mineralized fiber mats [58]. After washing with 1X PBS, the cell-containing mats were centrifuged in a Denville 2600 microcentrifuge (Denville Scientific, South Plainfield, NJ) at 6000 rpm for 2 min. RNA was extracted from each fiber mat by direct lysis of cells using an RNeasy Minikit (Qiagen-Cat No. 74104)

according to manufacturer instructions. The RNA isolated from each sample was transcribed into cDNA using the iScript® (Biorad Kit No. 170-8891) reagents and protocol according to the manufacturer specifications. The concentration and integrity of the cDNA was analyzed using the NanoDrop (Thermoscientific, Wilmington, DE). The samples for qPCR were prepared by mixing the Taqman Gene Expression Master Mix 2X (Life Technologies, Grand Island, NY), the Gene Expression Assay, and an appropriate volume of cDNA according to manufacturer protocol. Samples were, unless otherwise stated, run on an Eppendorf Mastercycler *realplex*² (Eppendorf, Hauppauge, NY) real-time PCR thermocycler; a hot start protocol was used holding the sample at 50°C for 2 min and 95°C for 10 min, followed by denaturation at 95°C for 15 s and annealing and extension at 60°C each for 1 min for 40 cycles. The quantitative analysis of gene expression was performed with the *realplex* 2.2 software (Eppendorf, Hauppauge, NY). The comparative C_T method or 2^{-C_T} was utilized in order to compare the expression of alkaline phosphatase to the endogenous control β-actin [40].

2.8 Statistical analysis

All cell experiments were performed in triplicate unless otherwise stated. Statistical analyses were conducted with either an unpaired Student's T test or an ANOVA with the GraphPad Prism 4.0 software (GraphPad, La Jolla, CA). Values are reported with a $p < 0.05$.

3. Results

3.1 Influence of nHAp and/or crosslinkers on fiber morphology

All solutions containing chitosan, crosslinker and various loads of nHAp in TFA could successfully be electrospun into fibrous mats. Figure 1 displays typical fiber surface morphologies while Figure 2 displays the diameter ranges of the spun fibers. The addition of either 1 wt% or 10 wt% nHAp alone yielded fibers with diameters that were within the standard deviation of pure chitosan fibers (133 ± 53 nm) [30], however, agglomeration and poor dispersion of nHAp particles (*ca.* 1 – 2 μm in diameter, $n = 50$ agglomerates) were observed. We have previously reported that the addition of the crosslinkers genipin and HDACS to the chitosan fibers increased the fiber diameters by 100% to 114% respectively [30]. Interestingly in this study, the addition of 1 wt% nHAp to either the chitosan-genipin or chitosan-HDACS solution produced fibers (125 ± 87 nm and 122 ± 57 nm, respectively) that not only had diameters within the standard deviation of the pure chitosan fibers [30], but had surface morphologies with lesser noticeable agglomeration of the nHAp particles (*ca.* 0.6 – 0.8 μm diameter, $n = 50$ agglomerates). The same surface morphology was observed at 10 wt% nHAp content, although fiber diameters were significantly higher than pure chitosan fibers ($p < 0.05$). The increase in fiber diameters correlated to an increase in nHAp contents. Agglomerations (*ca.* 0.8 μm diameter, $n = 50$) at higher mineral contents were also observed in electrospun PLGA with 1 – 20 wt% nHAp [41]. These findings suggest that nHAp disperses better in the electrospun chitosan-crosslinker fiber matrix. Moreover, the fiber diameters of the mats are within the range of the fibrous collagen (50 – 500 nm) [4] found in bone.

The addition of the white nHAp particles to the clear, light brown chitosan-TFA (pH 1) electrospinning solution resulted in a light brown and opaque mixture with increased pH of 2 – 3 due to the partial dissolution of the nHAp [42]. Although nHAp has been reported to survive and maintain crystallinity at low pH, unfavorable dissolution and a reduced number of nHAp crystals in the acidic solution are still possible [42, 43]. In order to reduce the exposure time of the nHAp to the acidic solution, the powder was mixed for only 2 min prior to electrospinning.

Hydroxyapatite found in bone is typically in crystal form and with its long crystal axis aligned parallel to the collagen fibrils. The favored crystalline structure of hydroxyapatite of biological origin has a stoichiometric ratio of 1.67 [44]. EDS scans of the surface of the fibrous composites yielded a Ca:P atomic weight percent ratio (Figure 2) in the range of about 1.11 – 1.70. Thus, the genipin and HDACS-crosslinked chitosan mats with 10 wt% nHAp have similar Ca:P ratios, suggesting that the addition of crosslinkers influenced the retention of the nHAp particles in the fiber matrix. Moreover, EDS estimates of the fluorine atomic weight percent in the crosslinked mats also suggest that during electrospinning at 10 wt% mineral content, chitosan and nHAp-particle interactions are more favored than the chitosan-trifluoroacetate interaction that was previously observed in pure chitosan fibrous mats [30].

3.2 Tensile properties

Figure 3 displays the typical stress-strain curves for all compositions while Figure 4 and Figure 5 display the corresponding representative FESEM micrographs of the mat and fracture surfaces taken after the unidirectional test and the mechanical properties E , σ_{UTS} and failure strain of the electrospun mats, respectively. Knowing that bone is a complex composite material with each material component contributing a characteristic mechanical property — the apatite phase provides bones with stiffness while the polymer phase provides toughness — the mechanical properties of the fibrous composites are important.

The results show that the different mat compositions result in different tensile properties and that they strongly correlate with the average fiber diameter of the mat, the amount of nHAp added and the type of crosslinker used.

Electrospun synthetic or biopolymer-based fibers typically have a low (~MPa) tensile modulus and strength [45–47]. In contrast, all nHAp-containing fibers here have a tensile strength and modulus similar or higher than those of other electrospun polymer-nHAp composite fiber mats reported in the literature [28, 46, 48]. In this study, fibers with smaller fiber diameters had a higher modulus and tensile strength. This trend was previously observed also in purely polymeric mats that were mechanically tested using 500 g load cell, strain rate of 0.02 s^{-1} , 21°C and 65% relative humidity. Neat chitosan mats, chitosan mats crosslinked with genipin and chitosan mats crosslinked with HDACS, spun without nHAp, had tensile moduli and strengths in the ranges of 257 – 512 MPa and 13.4 – 17.5 MPa, respectively [32].

3.3 Morphology of MLO-A5 cells

MLO-A5 cells are a preosteoblast line that are able to undergo spontaneous mineralization with the correct culture conditions and were first created and characterized by Kato *et al.* [49] [38]. The presence of collagen fibrils and extended cytoskeletal projections can indicate both the growth and the mineralization of the MLO-A5 cells. The IFC micrographs displayed in Figure 6 reveal the characteristic extended cell morphology when grown on all mineralized chitosan fiber mats after two days. Cells have spindle shaped cytoskeletal features, which is consistent with viability and proliferation. Multiple actin stress fibers are apparent for cells grown on all mineralized chitosan fibers. Appearance of a structured and layered network of MLO-A5 cells can be seen in the 1 wt% nHAp chitosan-HDACS and 10 wt% nHAp chitosan-HDACS nHAp fibers. This is less apparent with the 1 and 10 wt% nHAp genipin fibers. Further analysis of cellular morphology using FESEM reveals morphological differences between cells grown on the chitosan-genipin mineralized fibers compared to the chitosan-HDACS mineralized fibers (Figure 7). After two days post seeding, cells grown on 1 wt% nHAp chitosan-genipin fibers reveal cytoskeletal filopodia and collagen fibrils, which are clearly visible. In contrast, cells grown on the 10 wt% nHAp chitosan-genipin fibers display a more rounded morphology with less cytoskeletal projections. After nine days of growth on the 1 wt% nHAp chitosan-genipin fibers, a confluent multi-layer sheet of cells is apparent, whereas a rounded cell morphology occurs on the 10 wt% nHAp chitosan-genipin fibers (Figure 7). MLO-A5 cytoskeletal projections occur more frequently and are significantly longer ($p < 0.05$) with the 1 wt% nHAp chitosan-genipin fibers compared to the 10 wt% nHAp chitosan-genipin fibers. Cell growth on the mineralized chitosan-HDACS fibers show marked differences compared to the chitosan-genipin fibers. After two days post seeding, MLO-A5 cells display the extended morphology along with many cytoskeletal projections. An integration exists between the fiber scaffold and cytoskeletal processes for both mineralized fiber types. The length of cytoskeletal projections is not significantly different when comparing cells grown on either 1 or 10 wt% nHAp chitosan-HDACS fibers. After nine days post seeding, MLO-A5 cells are fully integrated within the fiber scaffolds and cannot be individually distinguished from one another.

3.4 ATP assay results of MLO-A5 cells

Results from the luminescent ATP assay reveal that MLO-A5 cells survive and proliferate when grown on all mineralized compositions of chitosan fiber mats. After two days post seeding, MLO-A5 cells possess similar ATP levels on all fiber mat compositions. However, after five days, marked differences can be seen among cells grown on the control TCP compared to both 1 and 10 wt% nHAp chitosan-genipin and chitosan-HDACS fibers. ATP levels begin to decrease for the 1 and 10 wt% nHAp chitosan-HDACS and 1 wt% nHAp chitosan-genipin fibers and this trend continues for the Day 9 time point.

3.5 qPCR analysis of alkaline phosphatase expression

In order to better understand the cellular response on a molecular level, the genetic expression profile of alkaline phosphatase (ALP) was determined *via* qPCR. Samples taken at the Day 2 time point did not yield quality RNA, however those on Days 3 and 4 yielded

measurable cDNA that could be characterized. ALP expression of MLO-A5 cells grown on mineralized fibers is very low compared to cells grown on the control TCP at the Day 3 time point (Supplementary Figure 1). Although the relative ALP level of chitosan-HDACS 1wt% nHAp is marginally above the control, this was determined to not be significant. By Day 4 however, there is a marked increase in ALP expression among cells grown on all mineralized fiber mats. Although these expression profiles look to be 100-fold times the control in some cases, due to the extreme variation in the data, these numbers were not determined to be significant. In addition, several samples were unable to be utilized for this experiment due to insufficient cDNA. (It is most likely that due to the high mineral content of each chitosan fiber scaffold, there was interference in the ability to obtain quality RNA for downstream qPCR applications. This may also explain the variability seen with regards to the qPCR profiles. In addition, these findings were consistent with the qPCR results found in our earlier work [58].

4.0 Discussion

In this study, mats with smaller fiber diameters and a 1 wt% nHAp content resulted in a higher tensile strength and modulus than mats with larger diameters and a 10 wt% nHAp mineral content. Similar to the effect of mineral addition on the properties of electrospun polymer-nHAp fiber mat reported in the literature, a very high load of nHAp to the fiber composite was found to reduce both modulus and tensile strength [28, 41], because at these concentrations the nHAp agglomerates act as defects rather than as a reinforcement. With 5wt% nHAp addition, the modulus of scaffolds consisting of PCL-chitosan-nHAp ($E = 4.56$ MPa) and chitosan-nHAp ($E = 0.67$ MPa) was reduced to 1.85 MPa and 0.45 MPa, respectively [50], indicating low stiffness at low mineral contents. Even at very low nHAp contents (2 wt%), no significant increase in tensile modulus was observed in 7 wt% chitosan fibers that were crosslinked with genipin (two-step) after electrospinning. It should also be noted that the previously reported electrospun chitosan-nHAp mats had larger fiber diameters (334.7 ± 119.1 nm) than the one-step crosslinked mats of this study (125 ± 87 nm) owing to the two-step crosslinking process with genipin. This most likely influenced the tensile modulus at the same 1 wt% nHAp contents in which the one-step genipin-crosslinked chitosan fiber mats displayed more than twice the tensile modulus ($E = 329 \pm 76$ MPa) in comparison to the two-step crosslinked mats ($E = 142.5 \pm 12.5$ MPa) [28]. Additionally, the two-step processed mats reported in the literature were measured in high hydration further lowering their measured tensile modulus.

The presence of the two different crosslinkers also influenced the tensile properties of the mats. Figure 3 displays typical stress-strain curves for the various mat compositions. Stress-strain curves for both uncrosslinked and crosslinked chitosan, both without nHAp, were included for comparison [32]. All mat compositions developed a neck during testing and exhibited an uneven mat surface after failure (Figure 4). Donius *et al.* [32] report four distinct patterns of mechanical performance of electrospun mats: (Type 1) brittle, combining high stiffness and strength with low failure strain; (Type 2) highly ductile – combining low modulus and strength with a high failure strain; (Type 3) intermediate stiffness, strength and failure strain and; (Type 4) brittle, with a high modulus, but considerably lower strength than the first type. In this study, chitosan-genipin with 1 wt% nHAp behaved like neat

chitosan-genipin mats – a Type 1 mat, while chitosan-HDACS with 1 wt% nHAp exhibited a Type 3 performance similar to that of chitosan. Additionally, genipin-containing mats, which are mats composed of a single layer and have higher fiber diameters than HDACS-containing mats which consists of 2 – 3 layers, were observed to have a higher tensile strength and modulus for both the 1 wt% and the 10 wt% nHAp loads. A similar fiber diameter and tensile property relationship was observed for chitosan-genipin and chitosan-HDACS mats without the nHAp [32]. This indicates that at lower nHAp addition, the crosslinker type dominates the mechanical properties and performance of the mats. In contrast, at high wt% nHAp the mineral content dominates the mechanical behavior of the mats. It should also be noted that at higher nHAp concentrations, the pH of the solution increases, leading to changes in the number of protonation sites in chitosan. Both genipin and HDACS have been reported to favor crosslinking under neutral [51, 52] and basic [33] conditions, respectively. Chitosan-genipin 10 wt% nHAp (Type 3) did not neither show embrittlement nor a gradual, step-wise fracture, a behavior that may be due to genipin self-crosslinking first (longer chains) before forming longer crosslinks with chitosan [51, 52]. It may also be due to the greater fiber alignment (*i.e.* fewer fiber-fiber contact points that are remaining) near the fracture area, which was not observed for the uncrosslinked and HDACS-crosslinked samples. The chitosan-genipin with 10 wt% mats also turned light bluish in color, indicative of the crosslinking [51, 52]. The slight increase in pH due to the addition of 10 wt% nHAp coupled by the reduced trifluoroacetate residues in the chitosan-HDACS fibers (indicated by the lower atomic wt% of F in Figure 2) may have favored the formation of more defects in mats leading to a more brittle structure, lower tensile properties and behaving similar to a Type 4 mat, such as chitosan-GA [32, 53]. Without crosslinkers, the chitosan with 10 wt% nHAp behaves like a Type 2 mat, with a low modulus and strength but a high failure strain ($20 \pm 9\%$) that is similar to that of the uncrosslinked chitosan [32]. It is suggested that future studies on these scaffolds be aimed at altering the ratio of chitosan, crosslinker and nHAp in the mats to create, over a large range and in a highly controlled fashion mechanical properties customized for a given application.

Cellular attachment and spreading to surfaces is dependent upon a variety of factors, with surface chemistry playing an integral role [54, 55]. Genipin will crosslink chitosan through the amine groups under acid conditions and HDACS will form urea linkages under basic conditions [56]. Each crosslinker will create a different chitosan surface chemistry, which may be one contributing factor of the cellular response. An additional factor to consider when analyzing the cellular response to the fiber mats is the scaffold's mechanical properties of the scaffold. When biomaterial scaffolds closely mimic the native mechanical ranges of bone, cells will grow into a mature phenotype as measured by the osteoblast marker alkaline phosphatase. Typically a minimal foreign body response is also achieved when implanted [57]. The mineralized chitosan-genipin fiber scaffolds have a higher tensile modulus and UTS compared to that of the mineralized chitosan-HDACS fiber scaffolds. However, the mechanical deformation behavior was found to be different between the crosslinkers. Where chitosan-genipin mineralized fibers deform as a Type 1 behavior, chitosan-HDACS fibers deform as a Type 3 behavior. Results of the tensile test have shown that chitosan-HDACS fibers deform and reveal many layers, whereas chitosan-genipin fibers deform and reveal one layer. The multi-layer structure of the mineralized chitosan-HDACS fibers may provide

a more favorable mechanical environment for the MLO-A5 cells. Finally, the tensile properties of all chitosan mineralized scaffolds are comparable to that of bone periosteum, which is the dense fibrous material that covers bone [58]. Bone periosteum from various bone regions of different test animals were shown to have elastic moduli of $E_{toe} = 0.43 - 1.93$ MPa, $E_{axial} = 26 - 230$ MPa and $E_{circ} = 4 - 96$ MPa [58]. Moreover, periosteum has been shown to facilitate and modulate growth of osteogenic cells deep in the fiber layers for bones that are not only young or growing, but also for older tissue that has survived trauma [58–60].

Orriss *et al.* have demonstrated that the biological mineralization pathway of maturing osteoblasts can be inhibited by endogenous ATP [61]. Although ATP is typically used as a marker for cellular metabolism, it is also an important cell signal receptor for a variety of biological pathways, including bone mineralization. Therefore, the decrease in ATP levels on both mineralized chitosan-HDACS fibers as well as 1 wt% nHAp chitosan-genipin, may be indicative of more mineralization occurring on these fiber compositions. As time on the fiber scaffolds increases, the MLO-A5 cells begin to produce more of their mineralized matrix, which would coincide with a decrease in ATP levels. These results are consistent with the FE-SEM and IFC images from Figures 6 and 7, as cells appear to grow best on both mineralized chitosan-HDACS fibers and 1 wt% nHAp chitosan-genipin. There was a decrease in cytoskeletal projections and appearance of collagen fibrils with MLO-A5 cells grown on 10 wt% nHAp chitosan-genipin fibers, which may coincide with the observed higher ATP levels. With these low ATP levels, we expected to see a large ALP expression difference with the cells grown on the mineralized CS fibers compared to that of the control TCP. However, this result was not witnessed, ALP expression was only slightly larger (Supplementary Figure 2). These results were consistent with Beringer *et al.* chitosan-HDACS fibers [58], and indicate that a panel of biological assays must be utilized in order to truly understand cellular response to a newly created biomaterial.

4. Conclusion

In this study, we have introduced the fabrication of electrospun chitosan mats that are, in a one-step process, crosslinked with genipin or HDACS and loaded with up to 10 wt% nHAp for potential bone tissue engineering. Microstructurally, the scaffolds with 10 wt% nHAp content have fiber diameters that are similar to collagen fibers in bone and have nHAp particles that have survived the electrospinning out of a low pH solution. The type of crosslinker and the amount of nHAp loading influenced the tensile properties of the electrospun composite chitosan-nHAp fibers. Moreover, the tensile property values were similar to that of bone periosteum, the fibrous bone covering that facilitates bone cell growth and repair. All mineralized fiber mats demonstrated potential for use as a bone scaffold material, even though their ALP expression profile was inconsistent.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Representative FESEM micrographs of the various electrospun composite chitosan-nHAp fibrous scaffolds (scale bars 5 µm).

Figure 2.

(A) Fiber diameters of the various electrospun composite chitosan-nHAp fibrous scaffolds and, (B) their respective atomic weight percent ratio of calcium:phosphate and fluorine contents from EDS. Dashed horizontal line indicates the stoichiometric Ca:P ratio (1.67) of crystalline hydroxyapatite.

Figure 3.

Representative stress-strain curves of all electrospun chitosan fiber compositions. Dotted lines indicate fibers without nHAp [32], solid lines denote those with 1 wt% nHAp, dashed lines indicate fibers with 10 wt% nHAp. Black texts denote uncrosslinked mats; red texts are genipin-crosslinked, while blue texts are HDACS-crosslinked.

Figure 4. FESEM micrographs (scale bars are 5 μm) of the various composite electrospun chitosan-hydroxyapatite fibers.

Figure 5. (A) Tensile modulus (square) and tensile strength (triangle) and the (B) strain at break of the various composite electrospun chitosan-hydroxyapatite fibers. Horizontal dashed lines at 512 MPa, 19 MPa and 19% denote pure chitosan fiber's tensile modulus, tensile strength and strain at break as previously reported by Donius *et al.* [32].

Figure 6.

Representative IFC micrographs of MLO-A5 cells grown on various mineralized chitosan fibrous scaffolds after 2 days.

Figure 7.

Representative FESEM micrographs of MLO-A5 cells grown on various mineralized chitosan fibrous scaffolds. (a) chitosan-genipin 1 and 10 nHAP (b) total cytoskeletal projection histogram (c) chitosan-HDACS 1 and 10 nHAP (d) total cytoskeletal projection histogram histogram distribution of cytoskeletal projections (b, d).

Figure 8.

ATP assay of MLO-A5 cells seeded upon mineralized chitosan fiber scaffolds. These values are average $\pm$ standard deviation of triplicate measurements.