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Fabrication, Optimization and Characterization of Synthetic Polymer Based Micro-Nano-Structured Composite Scaffolds for Bone Regeneration

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ABSTRACT

Various scaffold properties have been explored to understand the influence of physical and chemical properties on cellular behavior. Surface morphology is one property, which can be varied by modifying fiber diameter, and has been shown to play a role in cellular attachment, proliferation, and differentiation. The effect of fiber diameter on cellular proliferation and differentiation has offered varying results: some favor nanofibers and others favor microfibers in terms of their ability to encourage proliferation and cellular differentiation. In this study, the surface morphology was altered by modifying the fiber diameter of electrospun polycaprolactone (PCL). This study suggests that though higher attachment and proliferation rates are evident in scaffolds with lower average fiber diameters. However, when looking at cellular differentiation and mineralization, there appears to be a split in ideal fiber diameter ranges (400-800nm and 1.2-1.7μm). This leads to the recommendation that in future studies, a biomodal distribution of micro and nano ranged scaffolds be considered.

KEYWORDS osteogenic, differentiation, microfibers, nanofibers, fiber diameter

INTRODUCTION

Various scaffold properties have been explored to understand the influence of physical and chemical properties on cellular behavior. Surface morphology is a property which can be modified by varying fiber diameter and has shown to play a key role in cellular attachment, proliferation and differentiation.[3, 4] The analysis of cell morphology on electrospun fibers has indicated that projected cell area and aspect ratio are sensitive to the diameter and degree of fiber alignment of underlying scaffold [39]. Since cell shape and cell function are interdependent [39, 40], the architecture of electrospun scaffolds may affect cell phenotype.

The effect of the fiber diameter on cellular differentiation and proliferation has offered conflicting results: some in favor of nanofibers and others in favor of microfibers. Generally, fibers with diameters less than 1μm are termed as nanofibers. Nanofibers provide a connection between the nano and the macroscopic objects.[3] Due to their extremely high surface to mass ratio, nanofibers possess several novel properties such as low density, high pore volume, variable pore size and exceptional mechanical properties.[3] Studies have also shown that nanofibers create a pore size smaller than a cellular diameter will not allow sufficient cell migration within the structure. Additionally, the small size of the fibers tends not to maximize the points of cell attachment which is a negative effect on the expression of several factors and on cell spreading and differentiation. [8]

Studies with microfibers show that cells are able to use the empty surface of the fibers to effectively proliferate. Takasahi and Tabata suggest that the number of hMSC attached to the

non-woven fabrics increased relative to fiber diameter due to the fiber diameter being smaller than the size of cells (about 10 μm).[13]

In this study, the surface morphology was changed by altering the fiber diameter of electrospun polycaprolactone (PCL). It is hypothesized that when comparing a varying range of fiber diameter scaffolds (from nanometer to micrometer), there is a range of fiber diameters which is more ideal to promote osteogenic attachment, proliferation and differentiation.

This study has the primary objective to investigate the attachment, alignment, proliferation and osteogenic differentiation rate of hMSCs on varied fiber diameter scaffolds. In order to do this, as a prerequisite, an optimization study was done to vary the range of fiber diameter scaffolds from nanometers to micrometers.

PCL scaffolds were chosen in this study due to the polymer's slow degradation rate and ability to maintain its morphology throughout cell culture.

EXPERIMENTAL

Fabrication of Polymer Scaffolds

PCL with an average molecular weight of 80,000 (80 kDa) was obtained from Sigma Aldrich (Milwaukee, WI) and dissolved in 2,2,2- Trifluroethanol (MW= 100.04) from ACROS organics under gentle stirring to obtain various %wt solutions.

Scaffolds were fabricated using electrospinning techniques. The polymer solution was delivered at a constant flow rate (see specific rate in table 1), Harvard Apparatus PHD 2000 syringe pump, (Holliston, MA) to a metal capillary (1.6 mm OD, 1 mm ID, 50 mm length, Cole-Parmer, Vernon Hills, IL) connected to a high-voltage power supply (Gamma High Voltage Research ES-30P, Ormond Beach, FL). Upon applying a 13kV voltage, a fluid jet was ejected from the capillary. As the jet accelerated towards a grounded collector, the solvent evaporated and a charged polymer fiber was deposited on the collector in the form of a non-woven fabric. The non-woven fabric was stored in a desiccator for several days, then cut into 10×10 mm^2 squares (approx. 1 mm thickness).

An initial 28 day pilot study was conducted with a series of nine different polymer %wt and solvent combinations, in which data was recorded pertaining to cell proliferation and alkaline phosphatase activity. From the results of the pilot study, five PCL polymer %wt and TFE solvent combinations were determined to show strong correlation between diameter, proliferation and early stage differentiation and were chosen to be investigated in a more detailed study. **Table 1** displays the fabrication parameters used to achieve different the various fiber diameters for PCL fiber matrices via electrospinning.

Cell Culture

Human mesenchymal stem cell (hMSC) culture hMSCs acquired from Lonza (Walkersville, MD) were expanded on 100 mm tissue culture dishes (CytoOne) incubated at 37°C and 5% carbon dioxide with growth media consisting of DMEM (high glucose, + glutamine) supplemented with 10% fetal bovine serum and 0.1% P/S. Media was changed every other day and expanded to passage 6 for use in all experiments.

Scaffold Sterilization, hMSC Seeding and Culture

Fiber matrices and PCL thin films were sterilized by incubation in 70% ethanol for 30 min followed by exposure to UV for 30 min on each side. Care was taken with 1×1 cm^2 fibrous matrices to place them in 48 well plates such that the corners turned up, allowing the scaffold to sufficiently cover the bottom of the well and ensure cell seeding did not spill to underneath the scaffold. Once placed into sterile tissue culture plates or dishes, scaffolds were incubated for 3 h in growth media to remove any residual alcohol and to wet the scaffolds. Scaffolds were seeded at a seeding density of 50,000 cells per scaffold in 50μL of media, incubated at 37°C for 3 h to ensure cell attachment, and then brought to 700μL of media per well. Media were exchanged after 24 h and replaced every other day thereafter. After 24 hours, basal media was replaced with osteogenic media (Basal media, with 200 μM ascorbic acid, 10 mM sodium, β-glycerophosphate and 10 nM dexamethasone) for half of the scaffolds for the duration of the study.

Cell proliferation (PicoGreenDNA Assay)

DNA content was quantified at day 7, 14 and 21 days, using PicoGreen® dsDNA assay.[14,15]

The cellular constructs were washed twice with PBS, transferred to new well plates and 1 mL of 1% Triton X-100 solution was added to lyse the cells. The well plates underwent three freeze-thaw cycles and the contents were thoroughly mixed with the aid of a pipette to extract cell lysate. A 125 µL sample of DNA was transferred into a new well plate to which 375 µL of component B, and 500 µL Component A were added. Well plates were covered with aluminum foil to prevent light exposure and incubated for 5 min. A BioTek plate reader was used to measure fluorescence (485 nm/535 nm). Optical readings were converted to DNA concentration using a standard curve.[16]

Alkaline Phosphatase Activity

Levels of ALP, an early osteoblast phenotypic marker, by hMSC's cultured on scaffolds at 7, 14, 21 and 28 days was evaluated using an ALP substrate kit. [17] A volume of 100 µL of cell lysate was transferred into a well plate to which 400 µL of P-NPP (para- nitro phenol phosphate) substrate and buffer solution were added and incubated at 37°C for 30 min. After 30 min, 500 µL of 0.4 N of sodium hydroxide was added to stop the reaction. The intensity of the color produced through the reaction is proportional to ALP activity. The optical density of the solution was measured at 405 nm using a BioTek plate reader. The results for ALP activity optical density were normalized to DNA content. [16]

Mineralized Matrix Deposition Assay

Mineralized matrix deposition by hMSC on the PCL scaffolds was evaluated as a marker of mature osteoblast phenotype using an Alizarin red staining method for calcium deposition.[17-19] This colorimetric analysis is based on solubilizing the red matrix precipitate with CPC to yield a purple solution. At 7, 14, 21 and 28 days of culture, scaffolds were washed with PBS and transferred into new well plates. Cellular constructs were fixed with 70% ethanol at 4°C for 1 h and stained with a 40 mM Alizarin red (Sigma) solution (pH 4.23) for 10 min at room temperature. After washing 5-10 times with distilled water to remove the adsorbed/absorbed dye, chemically bound red matrix precipitate was solubilized in 1mL of 10% CPC until color was stable. The optical density of the solution was read at 562 nm using BioTek plate reader. The results for calcium deposition were also normalized by DNA content determined in a companion DNA assay described in the cell proliferation section.

Scanning Electron Microscopy (SEM)

The morphologies of the non-woven fiber matrices were characterized by SEM. The polymer coated surfaces were sputter coated with gold using a Hummer V sputtering system (Technics Inc., Baltimore, MD) before viewing with SEM. The samples were viewed using JSM 6400 scanning electron microscope (JEOL, Boston, MA) operated at an accelerating voltage of 20 kV at various magnifications. The fiber diameters were determined by (Image J, NIH) measuring the diameters of randomly selected fibers at different locations on the sample (n = 3). In each location 100 different fibers were selected for measurement.

Cellular Alignment

Live/Dead Assay Kit (L3224, Invitrogen) was used to visualize live and dead cells using confocal microscopy at various cell culture points. Cellular constructs were washed twice with warm PBS and then incubated with calcein-AM (2 μM) and ethidum homodimer-1 (EthD-1, 4 μM) and viewed under confocal microscopy at ex/em: 494/517 nm (calcein-AM, green) and 528/617 nm (ethidium homodimer-1, red). In brief, calcein AM enters live cells and reacts with intracellular esterase to produce a bright green fluorescence, while ethidium homodimer-1 enters only dead cells with damaged membranes and produces a bright red fluorescence upon binding to nucleic acids.

To investigate the changes in directional orientation of hMSC's seeded on the scaffolds, Fourier component analysis for directionality was performed on **Figure 4**, using the ImageJ plug-ins 'Directionality' created by Jean-Yves Tinevez (http://pacific.mpi-cbg.de/wiki/index.php/Directionality). Fiber matrices were imaged on day 7 using a BioRad Radiance 2100 Multiphoton/Laser Scanning Confocal Microscope (LSCM) and a Nikon Eclipse E600 Fluorescent Microscope at different magnifications.

Statistical Analysis

All results were first evaluated using one-way analysis of variation (ANOVA) followed by Tukey's HSD (Honestly Significant Differences) analysis of the differences between groups with a confidence range of 95%.

RESULTS AND DISCUSSION

Fiber Diameter Distributions

PCL was prepared at 12, 15, 17.5 and 20 %w/v concentrations and electrospun into fiber matrices having 80% of fiber diameters in the range of 200-400nm , 400-800nm, 1.2-1.7µm, and 1.8-2.2 µm, respectively (**Table 1**). Fiber matrices were named as Nano1, Nano2, Micro1 and Micro2 based upon increasing fiber diameter for identification purposes throughout the study. This range of fiber diameters can be seen in **Figure 1**. Electrospun nonwoven fiber matrices of thickness between 0.38 to 0.42 mm were used in this study. The fiber diameters of Nano1, Nano2, Micro1 and Micro2 are significantly different from one another **(Fig. 1).** Small fiber diameter accounts for higher surface area, higher tensile properties, lower porosity and lower wettability [41, 42].

SEM micrographs of fiber matrices of varying fiber diameter and the histographs to show the amount of varying fiber diameters is presented in **Figure 2**. The images display a bead-free morphology, indicating relatively good polymer stream stability**.** The fibers appeared to be smooth, and while there were some changes in diameter, the fiber morphology was generally consistent with the %w/v concentrations. The quantitative and qualitative observations were indicative of stable electrospinning conditions.

In a study done by Dietzel, et. al., the team reported a bimodal fiber diameter distribution in the micro/nano range in polymer solutions of 8 %wt poly(ethylene oxide) (PEO) or higher dissolved

in water.[21] This trend can be observed in the current study as well, with Micro1. Dietzel and team suggest that that the secondary population observed in the bimodal distributions of the electrospun fibers was formed due to the in-flight splitting or splaying of electrospinning jet before reaching the collector plate. [21] Overall the distribution of our fibers was consistent with those of others in the literature. [5, 8,24,25,29,30]

Bone derives its mechanical properties from a combination of structural designs in the nanoscale to macroscale range, with a precise interface between different components. Its intrinsic resistance to breakdown originates from different levels of hierarchical organization[43]. Plasticity and bone strength come from the smallest length scales, while larger length scales toughen bone by shielding the growing crack [43,44]. This well balanced micro-nano relationship is what needs to be understood to create a scaffold that allows for plasticity and protection.

Cell Attachment

Substrate micro- and nano-topography, independent of substrate biochemistry, appears to have an effect on cell behavior. Martinez et. al. reports on a series of studies which supports the trend that substrate topography (with micro- or nano-features) has direct effects on cell attachment, cell orientation, morphology, and cytoskeleton arrangements.[7]Anselme and team define adhesion as a phase, which occurs rapidly and involves events like physico-chemical linkages between cells and materials involving ionic forces, Van Der Walls forces, etc.[22]

Cell attachment was qualitatively assessed by SEM. **Figure 3a-d** demonstrates how the smallest nano fiber diameter matrices, Nano1, affected cell attachment in the first 24 hours. **Figure 3e-h** demonstrates how Micro2 affected cell attachment in the first 24 hours.

At 1 hr post seeding, cells on the nanofiber matrix appeared to remain on the surface while seemingly to have penetrated into the Microfiber matrix. This could suggest that the cells were able to infiltrate into the microfiber scaffolds, due to the larger pore size. It has been shown that the mean pore radius of electrospun matrices varies with fiber diameter [47]. For example, a 100-nm fiber diameter yields a mean pore radius of less than 10nm at a relative density of 80%. The comparative size of a rounded cell (ranging from 5 to 20 μm) shows that such small pore sizes will obstruct cellular migration.[45,46]

At 6 hr postseeding, cells appeared to have attached to the surface of the nanofiber matrix, potentially because smaller-diameter fibers offer more specific surface area [24,47]. Thus, offering a larger number of available focal adhesion points for cell attachment.

At 12 hours post seeding, it can be observed that the cells of the nanofiber matrix follow the phenomenon of stereotropism or physical guidance[10,20]. Images show that the fiber architecture guided the development of cell growth; however no additional data was collected to support this theory.

Within 24 hrs the cell attachment was observed to be relatively uniform, in both Nano1 and Micro2, indicating that saturation for cell attachment had been reached, and in the end the total number of cells adhering to the scaffold may not be significantly different. Cells on Nano1

matrices over time showed spreading and alignment in a particular direction. On the Micro2 scaffolds, cells appear to show a spread morphology, but no particular alignment was evident. In general both fiber matrices appear to have a well spread morphology over 24 hours. This shows the limitation of the SEM qualitative analysis, the inability to quantify the amount of cell attachment. However, the quantity of cells adhering may not be of concern since the quality of this adhesion will influence their morphology and their capacity for proliferation and differentiation.[23] In general, cells with a low motility form strong focal adhesions while motile cells form less adhesive structures. An intermediate level of attachment force induces a maximal migration rate.[23]

Cell Alignment

Microscopic examination of cell morphology following 7 days of seeding on the scaffolds revealed that cells on Nano1 appeared to be more aligned than cells on Micro1 (**Figure 4**). Micrographs help to visualize cells, which appear to be spread out, but aligned in one direction on Nano1 matrices **(Figure 4a**). On Micro1 scaffolds, cells appear to show a spread-out morphology but no particular alignment is evident (**Figure 4b**).

The preferred orientation cells on Nano1 and Micro1 was inferred using Fourier Spectrum analysis, shown in Figure 5. For a square image, the software slices the image into square pieces, and computes their Fourier power spectra. The latter are analyzed in polar coordinates, and the power is measured for each angle using the spatial filters proposed[48]. Images with completely isotropic content are expected to give a flat histogram, whereas images in which there is a preferred orientation are expected to give a histogram with a peak at that orientation. In **Figure**

5a the Nano1 scaffold shows cells with a significantly preferred orientation at 50º. In **Figure 5b** the Micro1 scaffold shows no significant orientation though there are small peaks at 75º and 100º.

Noting that the average diameter of Nano1 is substantially smaller than the dimensions of a cell (about 10µm in diameter [13]), hMSCs cannot spread in an unrestricted manner on fiber substrates, as they would on a planar surface. As a result, the intracellular cytoskeletal structure of hMSCs is likely altered as compared with planar surfaces, most notably resulting in cellular alignment along the dominant fiber axis[39]. Clark and team suggest that nanometer scale topography influences cell orientation on the substrate[49, 50]. During cell orientation filopodia provide details of the underlying surface nanometer scale topography. However, the recognition of the nanometer scale topography and how this information is signaled and interpreted by the cell is still an area of active investigation[43]. S.D. Subramony and team suggest that though cell alignment is visible on nanofibers, from their findings on PLGA, additional factors such as differentiation media or mechanical stimuli is needed for differentiation. Thereby demonstrating that both biomimetic architecture and physiologic stimulation is needed to control MSC differentiation and guide tissue healing without the addition of growth factors [51].

Cell Proliferation

Electrospun fibers due to their resemblance to the natural ECM, have been shown to affect cell growth and differentiation. [37] HMSCs were seeded on electrospun nanofibers. The greatest number of cells was found on Nano1 and Nano2 in both osteogenic (**Figure 6**) and basal (**Figure7**) media. Nano1 and Nano2 showed significantly higher proliferation rates than Micro1

and Micro2 at day 21. In osteogenic media, Nano1 and Nano 2 experienced a 70% and 40% increase in proliferation, respectively from day 14 to 21. Human MSCs showed significantly higher proliferation on PCL electrospun matrices having fiber diameters in the range of 398± 208 (Nano1) and 625 ± 193 (Nano2). Cell numbers increased on all samples till day 21, with the exception of Micro2 at day 14, which can be accredited to an initial lower seeding density or an overall limitation of the DNA assay extraction process, which limits the access and analysis of cells that may have infiltrated into the scaffold.

A trend can be noted in Figure 4 and Figure 5. An initial proliferation rate on nanofiber matrices that is 2 times higher than that of microfiber matrices, in both basal and osteogenic media. Takahaishi and Tabata[13], as well as Elias[23] and team noted the trend of high cellular proliferation on nanofibers, suggesting that nanofibers have a larger surface area-to-volume ratio that allow for initial cell attachment and proliferation. Chen and associates found that higher cell growth rates occurred on scaffolds that have an average fiber diameter of 428nm, but also noted an exception.[24] Chen and team note that when average fiber diameter changed from 1,051 to 1,647 nm, the proliferation rate increased, a value within the Micro1 range.[24] These results are also consistent with Badami and team who reported a higher cell density on larger diameter poly(D,L-lactate) fibers (2.1μm) than on smaller diameter fibers (.14μm). Chen and Badami both suggest that large-diameter fibers permitted more cellular infiltration than smaller-diameter fibers, although no proof of altered pore size or other mechanism of infiltration was offered [24,25]. In this study, Micro1 scaffolds do not exceed the proliferation rates of Nano1 or Nano2, but are significantly higher than Micro2 at day 21 and 28, in both basal and osteogenic media. Whether

bimodal distribution in fiber diameter (observed for 1.2-1.7μm scaffold) plays a role needs to be further explored.

Cellular Differentiation

ALP is a key component of bone regeneration due to of its role in the formation of apatitic calcium phosphate and it is an early indicator of immature osteoblast activity.[27, 28] Although cells in several tissues—liver, kidney, placenta, etc.—generate the enzyme, elevated levels of ALP in bone tissue typically are observed several days prior to neo-mineralization and during the initial phase of bone matrix deposition. [28, 31, 32] The early stage of osteogenic phenotype expression by hMSCs cultured on PCL scaffolds was evaluated by measuring the ALP activity. Normalized ALP expression with DNA content with osteogenic media is presented in **Figure 8**. A significantly higher level of ALP activity was observed on Nano2 and Micro1 matrices. These higher levels at early time points are indicative of early mature osteoblast phenotype expression on PCL scaffolds.

There was a statistically significant difference in ALP content at day 14 between Micro1 and all other concentrations. This suggests there is a microfiber range that can be conducive to enhance osteogenic differentiation, however no larger fiber diameters were used in this study to support this.

The trend of elevated ALP activity found in Nano2 and Micro1 can also be found in the basal media graphed in **Figure 9**. This significant trend follows an peak of ALP at day 14 followed by a decrease in activity for all fiber diameters. Tukey's analysis does not show a significant difference between Micro1 and Micro 2 fiber ranges at day 21 or 28; nor does it show a significant difference between Nano1 and Nano2 fiber ranges at day 21 or 28.

Following the osteoblastic differentiation model reported by Stein and Lian, cells proliferate up to 7–14 days and then start to secrete ECM proteins and produce early differentiation markers, such as ALP from day 7. [29, 30] When studying osteogenic differentiation for marrow stromal cells, Ma and team addresses this trend, noting that the maturation phase of cells generally lasts 8 and 12 days.[33] Once the mineralization phase begins, the ALP level starts to decrease while calcium content starts to increase.[33]

Alizarin Red is used to detect calcium deposits (mineralization).[35] Mineralization patterns were examined using Alizarin-red calcium staining in **figure 10.** Staining results indicate that cells seeded on Nano2 and Micro1 matrices display accelerated mineralization compared to control surfaces (not pictured). Additionally, analysis shows significantly greater calcium and phosphorous peaks for mineral deposits on Nano2 and Micro1 matrices at day 21 and 28, indicative of greater amounts of both elements. No mineralization was detected for scaffolds in basal media.

There are contrasting studies on the effect of fiber diameter, on mineralization. In separate studies, Ruckh[31] and Tuzlakoglu[5] claim that a mean PCL fiber diameter of 372 nm +/- 179 nm[31],

or a starch/polycaprolactone average of 400nm[5] fiber diameter allows for ideal cell infiltration and three-dimensional mineralization. Takahashi and Tabata conducted fiber diameter study with hMSCs on PET to suggest the ideal range of 9.0–12.0 μm.[13] Martinez et. al. also notes this issue of the lack of general trends due to the absence of systematic studies on this issue, as most of the reports found in published literature dealing with micro- and nano- structures and cell culture have been written by technology groups and are focused on microscopy observations.[7] As a consequence, cell mineralization is a parameter that is not always measured.

In this study, there was a split in results, showing that Nano2 and Micro1 showed a significantly large increase in mineralization by day 28.

A possibility for the positive results in ALP and mineralization for the Micro1 fiber matrices could be due to its bimodal spread. In comparison to the other bimodal fiber matrices, Micro 1 included nanofibers in the 400nm to 800nm, the range suggested as ideal from Ruck[31] and Tuzlakoglu[5]. This subset of nanofibers within the microfiber scaffold potentially added value with the high surface to volume ratio and in turn aided in initial attachment, prolifration and differentaition.

Chen and team address the possibility of a bimodal fiber distribution aiding in the positive results of their 1647nm PCL scaffold, but suggest further studies to ellucidate this phenomina.[24] In the case of multipotent mesenchymal stem cells (MSCs) a certain amount of nanometer scale disorder combined with micrometers has been found to stimulate MSCs to produce bone minerals in vitro in the absence of any osteogenic supplements [43,52,53]. Tuzlakoglu and team has

done indepth work on the bimodal approach. They suggest his bimodal approach is a way for a scaffold to mimic the physical structure of ECM for bone tissue regeneration while simultaneously providing the macro support that cells require. The team produced Nano- and microfiber combined scaffolds with starch/poycaprolactone. The study with human osteoblastlike cell line (SaOs-2) and rat bone marrow stromal cells demonstrated that the presence of nanofibers influenced cell shape and cytoskeletal organization of the cells on the nano and micro-combined scaffolds as well as cell viability and alkaline phosphatase activity.[44,45,46]

CONCLUSION

The last two decades have seen a tremendous level of fundamental research and development into nanotechnology. Recent developments in material science, engineering, biotechnology, and biomedical fields have clearly demonstrated the many potential applications of nanotechnology [43,54,55]. The basis of this intense nanotechnology-based research is derived from the fact that nanoscale matter can have significantly different properties than its bulk counterpart [43,56,57.] From a biomedical point of view, the cell is the basic unit of a biological system and every organism either consists of cells or is itself a single cell [43,58]. While cells are generally in the micrometer-size range, their component structures and associated environment are generally in the nanometer to submicrometer range [43].

The objective of this study was to investigate the attachment, proliferation and osteogenic differentiation of hMSC in the non-woven fabrics prepared from PCL fibers with varying

diameters. Altering the fiber diameter raises an interesting question: which will support enhanced cell growth, a scaffold with smaller fibers with more surface to volume ratio, or a scaffold with much larger fibers but significantly more room for cells to multiply and grow?

Many studies have analyzed the effect of varying fiber diameter size on osteogenic differentiation. Studies range from 117nm [25], 200nm [36], 372 nm [31], 1647nm [25], 2.1µm [26] to 12µm [13] with often conflicting results. In corroboration with Martinez, the reason for this lack of general trends could be the absence of systematic studies on this issue, as most of the reports found in the literature dealing with micro- and nano- structures and cell culture have been written by technology groups and are focused on microscopy observations.[7]

This study suggests that in osteogenic media conditions, fiber diameters matrices Nano2 and Micro 1 (400-800nm, 1.2-1.7µm, respectively) had positive results in terms of cellular differentiation. However, high initial attachment, proliferation rates, and specific alignment were observed in nanofiber matrices. This trend may influence future studies decide on a range of fiber diameters depending on the intended outcome. If optimal cell attachment is needed than smaller diameter ranges should be considerered. If differentiation is the intended goal, than micro fiber diameters should be considererd. However, differentiation cannot occur without attachment or proliferation and so a biomodal distribution of micro and nano ranges should be also considered. This study also shows that fiber diameter alone has an effect on cellular proliferation but little effect on osteogenic differentiation without the combination of other factors, such as differentiation media.

Electrospun scaffold architecture is an interesting challenge, in that there are currently few effective means to vary independently the three most basic geometric parameters: porosity, pore diameter and fiber diameter.[36-39] Future studies should examine a varying range of biomodal fiber diameters to help support or negate the theory that nanofibers within the microfiber scaffold could have aided in initial attachment, prolifration and differentaition.

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