Published in final edited form as:

ACS Appl Mater Interfaces. 2018 August 01; 10(30): 25069–25079. doi:10.1021/acsami.8b06386.

Electrospraying Electrospun Nanofiber Segments into Injectable Microspheres for Potential Cell Delivery

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Abstract

Nanofiber microspheres have attracted a lot of attention for biomedical applications because of their injectable and biomimetic properties. Herein, we report for the first time a new method for fabrication of nanofiber microspheres by combining electrospinning and electrospraying and explore their potential applications for cell therapy. Electrospraying of aqueous dispersions of electrospun nanofiber segments with desired length obtained by either cryocutting or homogenization into liquid nitrogen followed by freeze-drying and thermal treatment can form nanofiber microspheres. The microsphere size can be controlled by varying the applied voltage during the electrospray process. A variety of morphologies were achieved including solid, nanofiber, porous and nanofiber microspheres, and hollow nanofiber microspheres. Furthermore, a broad range of polymer and inorganic bioactive glass nanofiber-based nanofiber microspheres could be fabricated by electrospraying of their short nanofiber dispersions, indicating a comprehensive applicability of this method. A higher cell carrier efficiency of nanofiber microspheres as compared to solid microspheres was demonstrated with rat bone marrow-derived mesenchymal stem cells, along with the formation of microtissue-like structures in situ, when injected into microchannel devices. Also, mouse embryonic stem cells underwent neural differentiation on the nanofiber microspheres, indicated by positive staining of β -III-tubulin and neurite outgrowth. Taken together, we developed a new method for generating nanofiber microspheres that are injectable and have improved viability and maintenance of stem cells for potential application in cell therapy.

Graphical Abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsami.8b06386.

SEM images of electrospun NF segments; fluorescence images of nanofibrous PLGA–gelatin (1:1) microspheres; SEM images showing the adhesion of rBMSCs on solid microspheres and nanofibrous microspheres; and cell (rBMSCs)-laden nanofibrous microsphere aggregates injected into a PDMS channel (PDF)

mESCs on nanofiber microspheres after neuronal differentiation (MPG)

mESCs on solid microspheres after neuronal differentiation (MPG)

Notes

The authors declare no competing financial interest.

Keywords

electrospray; microdripping; electrospin; microspheres; stem cells

1. INTRODUCTION

Microspheres are at the forefront of drug delivery and tissue regeneration, as are three-dimensional (3D) porous scaffolds for the healing of large critical-sized defects.¹ Microspheres provide additional advantages over 3D scaffolds in that it can be cumbersome to fabricate 3D scaffolds for filling irregular shaped defects, while microspheres can be easily injected into any defect irrespective of the shape and geometry.² Further, the injectability of microspheres confers minimally invasive treatments as against the gross surgical procedures necessary for implantation of 3D porous scaffolds.³ The increased use of injectable microspheres also coincides with the advancements in biomedical imaging technologies for tracking them in the body postinjection.⁴ Apart from self-assembling/ionically cross-linked hydrogels, microspheres constitute the unique class of injectable biomaterials.⁵ However, the function of injectable microspheres is similar to 3D scaffolds in that they can also be used to deliver cells and growth factors/drugs to the site of injury for tissue regeneration.⁶ A host of natural polymers such as gelatin, chitosan, and alginate as well as synthetic polymers such as polylactic-*co*-glycolic acid (PLGA) and polycaprolactone (PCL) have been used for the fabrication of microspheres for various applications. The choice of the material used for microsphere fabrication is dictated by its biodegradability as necessary for drug delivery and sustained release as well as tissue regeneration. Also, the method of fabrication can critically affect the morphology, texture, and particle size distribution of the microspheres. For instance, to fabricate uniform-sized microspheres/microbeads with controllable porosity, a microfluidic device with optimized flow rates was successfully employed for PLGA microspheres.⁹ In another study, aerogel microspheres were fabricated by high-pressure spraying of cellulose nanofibrils through a steel nozzle into liquid nitrogen.¹⁰ To better control the particle size and distribution, electrospraying is a robust technology for the consolidation of nano/microparticles with a broad range of particle morphologies.¹¹ Further,

electrospray offers the advantage of a precise control over the particle size by manipulating the processing parameters such as the applied voltage, flow rate, and the distance between the spray needle and collector.¹¹ Particularly, in the dripping mode, a uniform size distribution of cell encapsulations within alginate microbeads was achieved.¹²

On similar lines, a broad range of microsphere morphologies have been fabricated by different methods, including solid, hollow, porous, and nanofiber (NF) microspheres and their combinations. The microsphere morphology can confer additional functionalities such as controlled and/or sequential growth factor/drug release from core-shell microspheres,¹³ enhanced cell viability and loading efficiency for porous microspheres,¹⁴ and tissue specificity as osteochondral repair for NF microspheres.¹⁵ The solid microspheres possess low cell carrier efficiency because of smaller surface area, whereas porous and NF microspheres can be loaded with more cells per microsphere arising from their larger surface area. The previously mentioned NF microspheres were fabricated by the self-assembly of star-shaped poly(L-lactic acid) (ss-PLLA)¹⁵ or gelation of chitosan coupled with microfluidics. These methods are limited by the polymer chemistry such as the necessity for specific surface functional groups and therefore selectively applicable to few biopolymers. These limitations necessitate the development of a fabrication method of NF microspheres, independent of the polymer chemistry and composition.

Apart from the application of injectable solid microspheres for the controlled delivery of therapeutics, solid microspheres bioconjugated to specific antibodies are being applied for the isolation, separation, and expansion of different cell types from their mixtures.¹⁷ A similar application of NF microspheres is envisaged with better cell separation and isolation efficiencies compared to solid microspheres. Another proven application of NF microspheres is the in vitro adhesion, proliferation, and maturation of chondrocytes as well as the in vivo cartilage formation and osteochondral repair induced by NF microspheres when injected together with chondrocytes.¹⁵ In another study, NF chitin microspheres mineralized with hydroxylapatite enhanced the adhesion of osteogenic precursor cell in vitro and in vivo healing of critical-sized radial defects in rabbits.¹⁸ In yet another notable study, NF microspheres formed by the self-assembly of PLA–poly(ethylene oxide)-based polymers were loaded with docetaxel and curcumin and such microspheres were efficacious in the treatment of colorectal cancer.¹⁹ All of the above examples are testimonials for the biomedical applications of NF microspheres. However, it must be reiterated that the previously mentioned NF microspheres were formed by self-assembly imposing a restriction on the composition of the NF microspheres.

In the present study, we report a novel method of NF microsphere fabrication by electrospraying of aqueous dispersions of electrospun fiber segments into a cryocoolant and explore their potential applications in cell delivery. This method is essentially a physical aggregation of NF segments into NF microspheres, which is largely independent of polymer chemistry and composition. Further, our approach is highly versatile for fabricating NF microspheres of a wide variety of compositions and morphologies for cell therapy in a minimally invasive way.

2. EXPERIMENTAL SECTION

2.1. Materials.

PCL (M_w = 80 000 and 45 000; Sigma), type A gelatin from porcine skin (300 g Bloom; Sigma), poly-lactic-*co*-glycolic acid (PLGA with 50:50 ratio of lactic acid–glycolic acid and ester-terminated, M_w $\approx$ 30 000–50 000 from LACTEL absorbable polymers), sodium alginate (Sigma), tetraethylene orthosilicate (Sigma), triethyl phosphate (Sigma), $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (Sigma), and glutaraldehyde (GA) (alcoholic solution).

2.2. Fabrication of Electrospun Polymeric and Bioactive Glass Fibers.

Electrospinning was employed to fabricate NFs of various compositions. The following compositions of NFs were electrospun for the present study—(i) PCL–gelatin in 1:1 ratio; (ii) poly-lactic-*co*-glycolic acid (PLGA with 50:50 monomer ratio)–type A gelatin in 1:1 ratio; (iii) poly-lactic-*co*-glycolic acid (PLGA with 50:50 monomer ratio)–type A gelatin in 3:1 ratio; and (iv) bioactive glass (Ca/P/Si) fibers. The PCL–gelatin and PLGA–gelatin electrospinning solutions were prepared by dissolving pre-estimated amounts of the polymers in hexafluoroisopropanol. The polymeric NFs were collected on a rotating mandrel at high and low speeds so as to obtain aligned and random NFs, respectively. The sol–gel-derived bioactive glass fibers were fabricated following the protocol reported in our previous work.²⁰ The typical electrospinning parameters were as follows—dc voltage = 15 kV, flow rate = 0.4–0.6 mL/h, and distance between the spinneret to collector = 10–15 cm. The polymer fibers were cross-linked by GA vapors from a 25% ethanolic solution overnight for $\sim$ 24 h.

2.3. Preparation of Segmented/Homogenized Short Electrospun Fibers from NF Mats.

The cross-linked polymeric NF mats were weighed for dispersion into water at predetermined concentrations. The weighed NF mats were segmented and/or homogenized using a cryostat and/or an ultrasonic homogenizer, respectively. In the former case, the NF mats were frozen in water at -80 °C and then cryocut at -20 °C with cutting thicknesses set to 30 and 50 μm to segment the aligned PCL–gelatin NFs. In the latter case, the random NF mats were scissored into tiny bits and then homogenized with a 20 kHz probe sonicator (Qsonica 500) equipped with a 1/8 in. tapered microtip probe under ice-cold conditions for 20 min using on/off cycles of 10/20 s and 20% amplitude. The aqueous dispersions of short fibers were subsequently used for electrospaying. For the inorganic bioactive glass fibers too, a similar homogenization protocol was followed.

2.4. Electrospaying of Segmented/Homogenized NF Dispersions.

All of the electrospaying experiments were performed in the dripping mode using aqueous short NF dispersions and aluminum (Al) foil immersed in liquid nitrogen as the ground collector for the particles. For the fabrication of NF microspheres, the fiber mats were typically dispersed in a nonsolvent such as water at a concentration of 20 mg/mL, whereas this was halved to 10 mg/mL for preparing porous NF microspheres. The typical electrospay parameters used were as follows: voltage = 8–10 kV, flow rate = 2.0 mL/h, and distance between the needle tip to collector/grounded electrode = 10 cm. The 21G syringe

needle with a nozzle diameter of 0.8 mm was used for all electrospraying experiments except for the coaxial electrospray. For the core-shell electrospray, the 20G needle with nozzle diameter of 0.902 mm was used as the outer needle and 27G with nozzle diameter of 0.4 mm was used for the inner needle and the two were indigenously connected with a Y-shaped tube to form a coaxial needle. For the core-shell microspheres, coaxial electrospraying was performed using dodecane as the core solvent at a flow rate of 0.4 mL/h, whereas the shell flow rate was maintained at 2.0 mL/h. After the electrospray microdripping, the frozen NF microspheres were immediately transferred to a freeze dryer and lyophilized for 24 h. Subsequently, the NF microspheres were thermally treated at 50 °C for 48 h to mechanically strengthen the particles. The core-shell microspheres were frozen and cryocut to confirm the hollow structure of the microspheres.

For the fabrication of solid microspheres, 5 wt % of lower molecular weight PCL ($M_w = 45\,000$) dissolved in dichloromethane was electrosprayed into an aqueous solution of 0.5 (v/v) % Tween 20 and 5 wt % gelatin. The electrospray parameters used were as follows: $v = 7.5$ kV, flow rate = 2.0 mL/h, and distance between the needle tip to collector of 20 cm. The aqueous solution was agitated by continuous stirring to prevent agglomeration of the particles. The collected PCL-gelatin microparticles were freeze-dried and cross-linked using GA vapors for 24 h.

2.5. Characterization of Microspheres.

2.5.1. Morphology and Size Characterization by Scanning Electron

Microscopy and Fluorescence Microscopy.—The morphology and particle size distribution of the microspheres were characterized by scanning electron microscopy (SEM) (FEI Quanta 200). The microspheres collected on Al foil were mounted onto a metallic stub using doublesided conductive carbon tape. The electrosprayed particles were sputter-coated in Ar atmosphere with a Au-Pd target at a peak current of 15 μ A for 5 min. The microspheres were subsequently imaged in the secondary electron mode using an accelerating voltage of 20–25 kV. The particle size distribution was determined using ImageJ software by the analysis of ~50 microspheres per sample group. For the core-shell electrosprayed microspheres, the particles were cryosectioned with a thickness of 20 μ m to demonstrate their hollow morphology. The autofluorescence of the cross-linked polymeric NFs was utilized to image the microspheres with a fluorescence microscope (Zeiss).

2.5.2. Bulk Density of the Microspheres.—The bulk density of the electrosprayed microspheres with different morphologies was determined by transferring a known weight of the microspheres into a volume-calibrated Falcon 15 mL conical centrifuge tube. This was followed by gentle tapping until the microspheres are settled down at the bottom and finally the volume occupied by the microspheres was measured.

2.6. Cell Culture Studies with Rat Bone Marrow-Derived Mesenchymal Stem Cells.

2.6.1. Isolation of Rat Bone Marrow-Derived Mesenchymal Stem Cells.—For the cell culture experiments, mesenchymal stem cells were isolated from the bone marrow of rat femur and tibia. The protocol for the isolation of rat bone marrow-derived mesenchymal stem cells (rBMSCs) was similar to that reported for isolation from mice.²¹ For the

rBMMSC isolation, the rats used were those being sacrificed following the completion of animal experiments approved by the Institute of Animal Care and Use Committee (IACUC) at the University of Nebraska Medical Center. Briefly, the hindlimbs of 12 week old rats euthanized by CO₂ asphyxiation were shaved to remove the animal hair and soaked in ethanol for 5 min. The skin and underlying fat tissue were cut open and scraped until neat bone samples were obtained. The rat bones were cut and washed in phosphate buffer saline (PBS) containing 1% antibiotic. Subsequently, a 23G needle was inserted into the bone marrow cavity and the rat bones were perfused with sterile low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS). The rBMMSCs were separated from the hematopoietic cells by their nonadherence to the cell culture polystyrene dish. The isolated rBMMSCs were maintained at 37 °C and 5% CO₂ in an incubator and cultured in a complete DMEM supplemented with 10% FBS as above for the cell adhesion experiments.

2.6.2. Adhesion of rBMMSCs on Microspheres.—For the cell adhesion and proliferation experiments, equal weights (~2 mg) of the solid and NF PCL–gelatin (1:1) microspheres were sterilized by soaking in absolute ethanol overnight and UV exposure for 24 h. The sterile microspheres were washed thrice in PBS and used for subsequent cell culture experiments. The cell adhesion and proliferation of rBMMSCs on the microspheres was determined by coculturing the microspheres with the rBMMSCs on agar-coated (0.1 wt %) 96-well plates. The microspheres were dispersed in complete DMEM and uniformly distributed in the wells of an agar-coated 96-well plate. As the cell culture experiments were being performed in static culture, a high seeding density of ~10 000 cells/well was used. After permitting cell adhesion for 24 h, the microspheres were transferred to different agar-coated wells and cultured for different time intervals. At designated time points, the cocultures of microspheres and rBMMSCs were harvested and fixed for confocal microscopy and SEM.

2.6.3. Fluorescent Staining of Cell Markers for Confocal Microscopy.—At time intervals of 1, 4, and 7 days, the microspheres were harvested by centrifugation at 1000 rpm for 1 min. The microspheres were washed thrice in PBS and the samples were fixed in 4% paraformaldehyde (PFA) at room temperature for 30 min. The fixed microspheres were washed thrice in PBS and immersed in a permeabilization and blocking buffer (1% bovine serum albumin and 0.1% triton in PBS) for 30 min. To stain the actin cytoskeleton, an appropriate dilution of the methanolic solution of Alexa Fluor 546 phalloidin (Invitrogen) in the blocking buffer was used and incubated with the fixed microspheres at room temperature in the dark for 30 min. The microspheres were washed with PBS and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for the nuclei. The images were acquired using a Zeiss LSM 800 with an Airyscan confocal microscope equipped with appropriate excitation and emission filters. Particularly, the z-stack images were orthogonally projected onto a single plane to determine the cell nuclei on each microsphere.

2.6.4. Sample Preparation of Rbmmsc–Microsphere Cocultures for SEM.—The rBMMSC–microsphere cocultures were harvested and fixed in 2% PFA–2% GA in PBS for 30 min at room temperature. The microspheres were subsequently washed thrice in PBS

and dehydrated in a gradient alcohol series of 30, 50, 75, 95, and 100% ethanol. The dehydrated microspheres were mounted on an Al stub and sputter-coated as previously for imaging under a scanning electron microscope.

2.6.5. Injection of Cell-Laden Microspheres into Polydimethylsiloxane

Channels.—To test the injectability of cell-laden microspheres and form tissue constructs in situ, the cocultured NF microspheres were injected into polydimethylsiloxane (PDMS) channels. The channels were created using a Sylgard 184 silicone elastomer kit (Dow Corning USA) with 21G needles (0.902 mm diameter) as templates. Briefly, the silicone elastomer and curing agent were mixed in a 10:1 volume ratio, respectively, degassed under vacuum to removal air bubbles, and poured onto a rectangular mold with three 21G needles as templates. PDMS was cured overnight at 60 °C in an oven. Subsequently, the needles were removed to generate channels of ~1 mm diameter.

For this experiment, a higher seeding density of 10^5 cells/well was used and the NF particles were cultured in a 0.1% agar-coated 96-well plate for 24 h. Subsequently, the cell-laden NF microspheres were injected into sterilized PDMS channels (~1 mm diameter) and cultured in the confined channels at 37 °C, 5% CO₂ for 3 days. Post the culture duration, the samples were fixed in 4% PFA overnight at 4 °C. The fixed samples were stained with Alexa Fluor phalloidin 546 and DAPI to visualize the actin cytoskeleton and nuclei, respectively. The images were acquired with the help of a Zeiss 710 confocal microscope.

2.7. Cell Culture Studies with Mouse Embryonic Stem Cells.

2.7.1. Mouse Embryonic Stem Cell Culture.—Frozen stocks of CE3 mouse embryonic stem cells (mESCs) were obtained from Dr. Younan Xia (Georgia Tech, Atlanta, USA). The mESCs were revived and cultured in T25 culture flasks coated with a 0.1% gelatin solution (Sigma-Aldrich, St. Louis, MO) in the presence of 5 μ L of 1000 U/mL leukemia inhibitory factor (LIF) (Invitrogen, Grand Island, NY) and 10 μ L of L-2-mercaptoethanol (β -ME) (Invitrogen, Grand Island, NY) to maintain their undifferentiated state. The cells were cultured in complete media consisting of DMEM (Invitrogen) supplemented with 10% new born calf serum, 10% FBS (Invitrogen), and 1% (v/v) EmbryoMax nucleosides and passaged at a ratio of 1:5 every 2 days.

2.7.2. Seeding of mESCs, Embryoid Body Formation, and Differentiation.—

The CE3 mESC suspension was adjusted to 1×10^6 cells/mL for culture with the microspheres. The CE3 mESCs were cocultured with the NF and solid microspheres in 0.1% agar (Sigma-Aldrich) solution-coated 100 mm Petri dishes for 3 days in the presence of LIF and β -ME. The undifferentiated CE3 mESCs were induced to form embryoid bodies (EBs) containing neural progenitor cells using the 4-/4+ retinoic acid treatment protocol, as reported previously. CE3 mESCs attached to the NF and solid microspheres were cultured in 0.1% agar solution-coated 100 mm

Petri dishes in complete media in the absence of LIF and β -ME for 4 days. Retinoic acid (Sigma-Aldrich) at 0.5 μ M was then added to the complete media for the final 4 days of culture. The media were changed every other day during the 8 day process. After EB formation, three-to-five cell-attached NF and solid microspheres were transferred to separate

wells of a 24-well plate (Corning, Corning, NY). To induce neural differentiation, 1 mL of neural basal media containing B27 supplement [Invitrogen, 1:50 (v/v)] was added to each well of a 24-well plate containing the EB-attached microspheres. The culture was continued for 14 days with periodic replenishment of the neural basal media supplemented with B27.

2.7.3. Immunohistochemistry of the Neural Marker.— β -III-tubulin (Tuj1) is a well-reported neural cell-specific marker.^{23,24} Therefore, immunostaining was performed using Tuj1 primary antibody to decipher neural differentiation of mESCs. After 14 days of EB coculture with the microspheres, the samples in each well of the 24-well plate were washed with 1 mL of PBS (Invitrogen) and then fixed for 30 min with 500 μ L of 4% PFA. Then, the cells were permeabilized in 400 μ L of 0.5% Triton-X in PBS for 10 min. The cells were blocked with 500 μ L of 5% normal goat serum (Invitrogen) in PBS for 30 min and incubated with Tuj1 (1:200) primary antibody overnight at 4 °C. After that each well was washed thrice with PBS at min 5 interval. Appropriate secondary antibodies (1:300 dilution) were applied for 1 h at room temperature and each well was washed thrice with PBS at 5 min interval. Finally, each well was incubated with 500 μ L of DAPI solution (1:2000) for 5 min and then washed thrice with PBS at 5 min interval. The fluorescence images of the samples were acquired under a Zeiss 710 confocal microscope.

2.8. Statistical Analysis.

All of the cell culture experiments were performed in multiple replicates ($n = 6$) and the data shown are the mean $\pm$ standard deviation of these experiments. The IBM SPSS software version 2.0 was used for performing the statistical analysis. For determining statistical significance, one way ANOVA with Tukey test was performed with a statistical significance set at $p < 0.05$, where p is the probability that there is no significant difference between the means of the compared groups.

3. RESULTS

Figure 1 is a schematic summary of the fabrication of NF microspheres from short fiber segments prepared by cryocutting and/or homogenization with a probe sonicator in a solvent (i.e., deionized water) that does not dissolve the fiber mat. The homogenized fiber dispersions were electrosprayed into liquid nitrogen in the dripping mode, followed by freeze-drying and thermal treatment to mechanically strengthen the microspheres. Finally, the microspheres were demonstrated for their utility as stem cell/progenitor cell carriers and their potential for the delivery of therapeutics.

3.1. Characterization of Short Electrospun NFs.

The short electrospun NF segments were prepared by cryocutting and/or homogenizing with a probe sonicator. The segmented NF dispersions were characterized by fluorescence microscopy and SEM. Typically, aligned PCL–gelatin NF mats were cryocut to segments of 30 and 50 μ m in length, whereas random fiber mats of PLGA–gelatin and bioactive glass fibers were homogenized using a probe sonicator. Figure 2A shows fluorescence images corresponding to the homogenized PLGA–gelatin (1:1) short fibers. The length distribution of the NF segments shown in Figure 2B was obtained by measuring the fiber lengths of

>250 fiber segments from 10 images. A median length of $\sim 20 \mu\text{m}$ was discerned from the analysis of the short fiber segments under the homogenization conditions (20% amplitude, on/off cycles of 10/20 s each, and 20 min of sonication time) indicated. By manipulating the homogenization parameters such as amplitude, frequency, duration of on and off cycles, and sonication time, it is possible to control the median size of the homogenized short fibers. Figure 2C,D shows the fiber segments of aligned PCL–gelatin (1:1) NF cryocut to 50 and 30 μm , respectively. The minor variations in the lengths of cryocut fiber segments could be due to small misorientation of the supposedly aligned NF mats. The rationale for using two different methods for the two polymer compositions lies in their glass-transition temperatures. The PLGA copolymers are glassy in nature and can be fragmented by homogenization because of their high glass-transition temperature, which is greater than the physiological body temperature of 37 °C.²⁵ On the other hand, PCL has been reported to possess a low glass-transition temperature of $-50 \text{ }^{\circ}\text{C}$ ²⁶ and hence cannot be fragmented by homogenization because of its viscoelastic behavior even under ice-cold conditions. Therefore, the PCL–gelatin fibers were segmented into short fibers by cryocutting, followed by dispersing the cryocut segments by homogenization. Figure S1A–C shows the scanning electron micrographs of homogenized PLGA–gelatin, PCL–gelatin cryocut to 50 μm , and PCL–gelatin cryocut to 30 μm lengths, respectively.

3.2. Effect of Electrospraying Processing Parameters on the Morphology and Size Distribution of Microspheres.

The morphologies and size distribution of the different microspheres fabricated by electrospraying were measured by SEM and fluorescence microscopy. On the basis of the established theory and experimental data showing that the particle size can be manipulated by varying the electrospray processing parameters such as applied voltage and flow rate,^{27–29} in the current study, the voltage was varied between 8 and 10 kV at a fixed flow rate of 2.0 mL/h to fabricate NF microspheres of different sizes. Figure 3 shows the morphology and size distribution of NF PCL–gelatin (1:1) NF microspheres. From the SEM images in Figure 3A,B, it can be observed that the particle size was more uniform between 400 and 450 μm along with a narrow size distribution of the microspheres at a lower voltage of 8 kV. This can be rationalized in that lower voltages facilitate larger and uniform droplet formation during electrospray in the dripping mode. Upon further increasing the direct current voltage to 9 kV, smaller droplets are formed, leading to smaller size of the microspheres with a rather broad size distribution ranging from 150 to 275 μm (Figure 3C,D). Further increase in the applied voltage to 10 kV did not significantly reduce the microsphere size, which ranged from 125 to 200 μm . The broader size variation at higher voltage could possibly result from combination of dripping and unstable spraying of the NF dispersions during electrospray. Sometimes, the clogging of the needle tip occurred because of the sedimentation of the NF segments. At such times, the needle was changed and/or the NF dispersion in the syringe was manually mixed. Although lower flow rates can reduce the particle size, we did not resort to this option as it would reduce the yield of the NF microspheres. On the other hand, higher flow rates caused greater aggregation of the microspheres, which distorted upon agitation to disperse them. Perhaps, a better engineering of the electrospray fabrication process can lead to the production of NF microspheres with uniform size. Nevertheless, a

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biomimetic NF surface topography can be seen for all of the microspheres and this is a more important factor from a tissue engineering perspective.³⁰

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Apart from controlling the size distribution of the NF microspheres, several morphologies of the microspheres could be achieved by electrospraying. Figure 4 highlights the different microsphere morphologies fabricated by electrospraying in the current study. Figure 4A corresponds to the regular solid PCL–gelatin microspheres. Taking cue from a previous report, a lower molecular weight of PCL ($M_w = 45\,000$) was used to obtain microspheres with spherical morphology. However, it was difficult to obtain solid microspheres with uniform size $100\,\mu\text{m}$ as electrospraying in the dripping mode resulted in microsphere aggregation, whereas the cone-jet spraying mode resulted in smaller size and broad size distribution. Therefore, the processing conditions chosen were at the border of the two electrospray modes. Figure 4B,C shows the regular dense PCL–gelatin NF microspheres and porous NF microspheres fabricated with high and low fiber densities of 20 and 10 mg/mL, respectively. This is analogous to the PLGA concentration-dependent fabrication of uniform beads of PLGA with controllable pore size from a water-in-oil-in-water emulsion flowing through a microfluidic device.⁹ Figure 4D denotes core-shell PCL–gelatin microspheres obtained by coaxial electrospray with a segmented PCL–gelatin shell and dodecane solvent as the core. A solvent immiscible with water and a freezing point above the freeze-drying temperature was necessary to prevent the collapse of the microspheres. Dodecane has a freezing/melting point of $-10\,^{\circ}\text{C}$ and thus satisfied both requirements. The hollow structure of the core-shell microspheres was observed after cryosectioning the frozen spheres as shown in the inset of Figure 4D. Depending on the flow rates of the core and sheath fluids, the shell thickness of the hollow microspheres can be controlled. Further, the needle gauges used for the coaxial electrospray were 20G (diameter of 0.902 mm) for the outer needle and 27G (diameter of 0.4 mm) for the inner needle. The large diameter of the outer needle led to larger-sized core-shell microspheres after coaxial electrospray. With regard to cell delivery, literature reports suggest that hollow NF microspheres exhibit better cell adhesion and proliferation as both the exterior and interior of the sphere are available for cell adhesion.¹⁵ However, in our study, the hollow spheres do not possess any cavity/opening on the surface for cells to migrate from the exterior into the interior of the sphere. Perhaps, the porous NF microspheres are more competent for cell delivery as they can permit greater cell infiltration but their mechanical stability was poor for long-term cell culture.

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In addition to obtaining multiple particle morphologies, it was also possible to fabricate NF microspheres from various organic polymer composite fibers as well as inorganic bioactive glass fibers. Figure 5A,B shows the SEM images of NF microspheres of PLGA–gelatin in 1:1 and 3:1 weight ratios, respectively. It was not possible to fabricate NF microspheres of pristine PLGA. This led us to conclude that a critical amount of gelatin is necessary to glue the NFs together during electrospray of electrospun NF segments. The mechanical stability of the NF microspheres was greatest for the polymer–gelatin ratio of 1:1. To fabricate PLGA NF microspheres, homogenized PLGA NFs at 10 mg/mL fiber concentration was mixed with 0.25 wt % of gelatin and electrosprayed (Figure 5C). In a similar manner, homogenized bioactive glass at 10 mg/mL was dispersed in 0.25 wt % of alginate to fabricate bioactive glass NF microspheres (Figure 5D). Thus, we demonstrate that a broad range of polymer

compositions can be successfully used for the fabrication of NF microspheres by electrospray microdripping of homogenized/segmented short fiber dispersions.

3.3. Porosity of NF Microspheres.

The bulk densities of the electrosprayed microspheres were calculated by measuring the volume occupied by a known weight of microspheres. Table 1 lists the bulk densities of the different microsphere morphologies fabricated by electrospraying. From the values listed in Table 1, a marked difference in the density of NF microspheres and solid microspheres may be noted for both PCL–gelatin (1:1) and PLGA–gelatin (1:1) compositions. The NF microspheres were 50 times lighter for the same volume occupied by the solid microspheres. This indicates the highly porous nature of the NF microspheres as compared to the solid spheres. Further, the bulk densities of NF microspheres were on the order of 5–10 mg/mL, which is similar to that recorded for cellulose nanofibril-derived aerogel microspheres fabricated by high-pressure spraying into liquid nitrogen.¹⁰ Among the NF microspheres, no significant variation in the bulk densities of the hollow and porous NF microspheres was noted. These porosity characteristics led us to choose the solid and NF microspheres for comparative cell culture study.

3.4. Enhanced Adhesion, Proliferation, and Inject-ability of rBMMS-C-Laden NF Microspheres.

The in vitro adhesion and proliferation of rBMMS-Cs was examined on NF microspheres and compared with the solid microspheres. As anticipated, the extracellular matrix (ECM)-mimicking nature of the NF microspheres promoted the initial cell adhesion as well as proliferation of rBMMS-Cs in comparison to the solid microspheres. From the cell morphologies on the NF microspheres, it is evident that the adhered rBMMS-Cs were well spread on the 3D NF topography as against the smooth surface presented by the solid microspheres. Figure 6 presents representative confocal images showing the attachment and multiplication of rBMMS-Cs on solid and NF microspheres at different time intervals under static culture. The statistics presented in Figure 6 reveal a higher number of cell cargo per microsphere payload for the NF microspheres as compared to the solid microspheres. Interestingly, the rBMMS-Cs seemed to prefer to occupy the voids or spaces between adjacent microspheres in the case of solid microspheres as shown in Figure S3. It may be noted that the mechanical stability of the NF microspheres was poor under the shear applied in 3D spinner flask culture, necessitating static culture experiments.

The cell (rBMMS-C)-laden NF microspheres were injected into PDMS channels of ~1 mm diameter and cultured for 3 days. Because of the high cell seeding density and proliferation of the rBMMS-Cs, the injected microsphere aggregates led to the formation of in situ microtissue-like structures as shown in Figure S4. This demonstrates the injectability of the cell-laden NF microspheres into irregular-shaped defects for eliciting tissue regeneration in a minimally invasive manner.

3.5. Neural Differentiation of mESCs on NF Microspheres.

Previous work from our group demonstrated that the aligned PCL 2D NF mats facilitated the 4–/4+ retinoid acid protocol-induced neural differentiation of embryoid bodies prepared

from similar CE3 mESCs.²² Further, several reports of neural differentiation of mESCs on NF matrices already exist in the literature.^{32–34} In all of the aforementioned references, the neural differentiation of mESCs on NF matrices was ascertained by positive immunostaining for nestin and β -III-tubulin (Tuj1), reverse transcription polymerase chain reaction analysis of neural specific genes such of nestin, Tuj1, MAP2, SOX1, and PAX6, and electrophysiology experiments. Therefore, in our study too, it was expected that the expression of neural marker genes would be consistent with positive Tuj1 staining as reported in previous studies. Hence, we have undertaken to evaluate neural differentiation of mESCs with Tuj1 as a neural marker in this study. With this hindsight, the differentiation of mESCs to neural lineage was compared on both the solid and NF microspheres. Figure 7A,B shows the confocal images of the mESCs on the NF and solid microspheres under neurogenic induction culture, respectively. The individual frames in Figure 7A,B shows the confocal images captured at distances of 15, 30, 45, and 60 μ m, in between the first and last frames of a recorded z-stack. Several neurite extensions with positive staining for Tuj1 (red fluorescence arising from antibody conjugated to Alexa Fluor 647), a neural differentiation marker, can be seen in the case of NF microspheres (Figure 7A). On the contrary, the number of cells adhered on the solid microspheres was meagre with only few cells exhibiting neurite protrusions with positive Tuj1 (β -III-tubulin) staining (Figure 7B). The image frames captured during the z-stack acquisition have been converted into separate videos, which have been provided in the Supporting Information (Videos S1 and S2). The higher cell adhesion of mESCs on NF microspheres is consistent with the higher adhesion and proliferation of rBMMSCs on similar spheres, reported in the previous section.

4. DISCUSSION

Stem cell therapy holds great promise for the regeneration of tissue defects/injuries, autoimmune diseases, and neurodegenerative disorders.³⁵ Although adult stem cells do not pose the risk of teratoma, immune rejection, and ethical issues, their isolation and expansion on two-dimensional (2D) polystyrene culture dishes different from their in vivo 3D niche environment weakens their paracrine functions and homing capability to the site of injury.³⁶ Further, the injection of the stem cells either systemically, intravenously, intra-peritonally, or locally into the tissue defect results in poor survival, migration to other tissues/organs than the target site, and consequent disability to form 3D tissues.³⁷ This has necessitated the development of stem cell carriers in the form of injectable hydrogels and microspheres. In this light, the biomimetic NF microspheres are perhaps the best choice as delivery vehicles for stem cells as their 3D NF topography recapitulates the ECM niche of adult stem cells.

For geometric- and regular-shaped defects, it is advantageous to culture stem cells in 3D porous scaffolds with pore interconnectivity and transplant the cell-laden scaffold into the defect.³⁸ Additionally, the NF topography in 3D porous scaffolds can better mimic the ECM of native tissue. Recent work from our research group has demonstrated that the NF architecture of 3D hybrid porous NF aerogels with bone ECM- like structure is crucial for neovascularization of the regenerated bone tissue in 8 mm rat calvarial defects.³⁹ Inspired by the 3D NF aerogels, in the current work, we demonstrate the fabrication of NF aerogel microspheres for injectable stem cell therapy into irregular-shaped defects. The minimal invasiveness of the injectable NF microspheres is perhaps a better bet compared to surgical

implantation of 3D porous scaffolds for stem cell therapy-based tissue regeneration. It may be noted that the NF aerogel microspheres are simply referred to as NF microspheres.

A large body of literature exists for the different types of porous microspheres with surface pores and internal pores fabricated from a broad range of precursor polymers including PLGA, PLLA, chitosan, and PCL utilizing porogens of inorganic salts, gelatin, sugar, and so forth.¹⁴ A water-in-oil-in-water emulsion generated by the combined flow of phases from three channels in the fluidic device was employed for the fabrication of PLGA microspheres with surface pores and hollow interior.⁴⁰ Using a similar fluidic flow of the water-in-oil-in-water emulsion, uniform PLGA microspheres with controllable pore sizes were fabricated.⁹ In contrast, the fabrication of NF microspheres has been limited to the self-assembly of ss-PLLA⁴¹ and phase separation of polyhydroxy-butyrate⁶ and chitosan/chitin derivatives.¹⁶ In this light, the current study demonstrates the fabrication of NF microspheres with a broad variety of polymer compositions by electro-spraying of homogenized electrospun fiber aqueous dispersions. The NF microspheres fabricated in the current study were conducive for the culture of undifferentiated bone marrow stem cells as well as neuronally differentiated ESCs. Further, the aerogel NF microspheres can form microtissue-like structures in situ, when injected into a PDMS microchannel. These results suggest that the NF microspheres can be applied as injectable scaffolds for stem cell-based tissue regeneration of irregular-shaped defects. However, the biodegradation of the PCL–gelatin (1:1) NF microspheres can be a concern for tissue engineering applications. Previous studies indicate that ~50% weight loss occurred over 1 week because of the erosion of gelatin from similar GA cross-linked PCL–gelatin (1:1) NF mats when immersed in PBS at 37 °C.⁴² On the other hand, PCL degradation was not detected in similar PCL–gelatin NFs even after 90 days of incubation in PBS.⁴³ Nevertheless, the PCL–gelatin (1:1) NF microspheres were used for the cell culture experiments only to demonstrate the feasibility of using such particles as cell microcarriers. Depending on the regeneration of specific tissue types, the composition of the NF microspheres can be tailored to adjust the degradation rate to match the tissue ingrowth. This study also demonstrated the fabrication of NF microspheres with several compositions [e.g., PLGA–gelatin (1:1), PLGA–gelatin (3:1), and PLGA–collagen–gelatin (2:1:1)]. In addition, our recent study demonstrated that 3D NF aerogels composed of PLGA–collagen–gelatin (2:1:1) were successfully applied for cranial bone regeneration in rats.³⁹ In that study, the PLGA–collagen–gelatin (2:1:1) NF aerogels underwent in vivo degradation within 4 weeks and were completely resorbed within 8 weeks.

Furthermore, the field of tissue engineering is currently undergoing a paradigm shift with regard to the treatment of tissue defects of irregular geometry and shape.⁴⁴ Minimally invasive injectable biomaterials coupled with stem cells/progenitor cells are preferred over painful surgeries that necessitate nominal postoperative treatment and care.⁴⁵ This class of injectable biomaterials is largely composed of hydrogels and microspheres. A recent dedicated review highlights the advantages of injectable biomaterial scaffolds over preformed scaffolds with defined shape and geometry, with a special focus on craniofacial and dental tissue regeneration.⁵ In particular, the application of injectable NF microspheres has largely been demonstrated for promoting chondrocyte function in vitro and cartilage repair in vivo.^{15,16} These studies show that the ECM-like physicochemical properties of the NF microspheres elicit superior cartilaginous activity in chondrocytes, when compared to

the solid microspheres. An interesting study illustrates the synergy of BMP-2 release from heparin-conjugated gelatin solid nanospheres encapsulated within hierarchical PLLA NF microspheres as an effective osteoinductive scaffold for healing 5 mm calvarial defect in vivo.⁴⁶ In another work from the same group, vascular endothelial growth factor release from heparin-conjugated gelatin solid nanospheres within PLLA NF microspheres promoted dental pulp regeneration in human teeth, subcutaneously implanted in immunocompromised nude mice.⁴⁷ A similar strategy was employed for transfection by a two-stage delivery of DNA polyplexes encapsulated in PLGA nanospheres, hierarchically embedded in NF PLLA microspheres. The injection of the above hierarchical system was effective against pathogenic tissue fibrosis and aided in support disc regeneration.⁴⁸ All of the aforementioned studies exploit the synergy of embedded solid nanospheres for therapeutic delivery within the ECM-mimicking topography of the NF microspheres for tissue regeneration.

Although injectable microspheres have been largely implicated in cartilage and bone tissue engineering, we want to emphasize their potential for application in neural and cardiac tissue regeneration. The NF microspheres have been shown to be compatible with mESCs, which differentiated into neurons in the neural induction media. Such cell-laden, injectable NF microspheres could potentially be used to treat brain stroke, neurodegenerative diseases such as Alzheimer's and Parkinson's disease, and cardiac disorders such as scar tissue formation following myocardial infarction. All of the aforementioned diseases are characterized by ischemia-induced cell death. Therefore, the replenishment of ischemia/hypoxia preconditioned stem cells/progenitor cells at the ischemic locations can enhance their survival and lead to tissue regeneration.⁴⁹ To this end, the NF microspheres in the current study have been demonstrated to be compatible cell carriers for different stem cell/progenitor cell types. Also, the current study is the first demonstration of combination of electrospray and electrospinning for fabrication of NF microspheres. In our future studies, we intend to increase the pore size of our NF microspheres by controlling the freezing temperature during electrospraying of the NF dispersions into microspheres. This can be achieved by using higher freezing temperatures that can generate larger pores in 3D NF aerogels, as demonstrated in our recent publication,³⁹ which is also consistent with the established mechanism of ice crystal growth during freezing of organic-inorganic dispersions.⁵⁰ Taken together, the NF microspheres developed in the current study could pave the way for better application of cell therapy in regenerative medicine.

5. CONCLUSIONS

The present study is the first report of fabrication of electrospun NF microspheres using electrospray. As against previous methods of phase separation and self-assembly of polymers with specific surface chemistry, our approach of electrospraying of aqueous dispersions of short electrospun NF segments into a cryocoolant can be applied to a broad range of polymer compositions, for different microsphere morphologies, and for the fabrication of particles of different sizes with narrow size distribution. The aerogel NF microspheres elicit enhanced proliferation and differentiation of stem cells in comparison to solid microspheres. Further, the injectable, cell-laden NF microspheres can form in situ microtissue-like structures when cultured in confined spaces within micro-channels. Taken

together, the injectable NF microspheres are promising cell delivery vehicles that can aid in tissue regeneration of irregular-shaped defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institute of General Medical Science (NIGMS) at the NIH (2P20 GM103480-06 and 1R01GM123081), NE LB606, Regenerative Medicine Program pilot grant and startup funds from the University of Nebraska Medical Center. The authors thank Janice A. Taylor and James R. Talaska of the Advanced Microscopy Core Facility at the University of Nebraska Medical Center for providing assistance with confocal microscopy.

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Figure 1. Schematic overview of the fabrication of NF microspheres from segmented/homogenized electrospun NFs and their applications for stem cell and drug/peptide delivery.

Figure 2. Morphology (A) and size distribution (B) of PLGA-gelatin (1:1) electrospun NFs homogenized with a probe sonicator. Aligned PCL-gelatin (1:1) NFs segmented to lengths of 50 (C) and 30 μm (D) using a cryotome. The electrospun fibers upon cross-linking with GA vapors exhibit autofluorescence.

Figure 3.

Variation in the particle size of PCL–gelatin (1:1) NF microspheres (G) as a function of applied voltage [8 kV (A,B); 9 kV (C,D); and 10 kV (E,F)] at fixed flow rate and distance between the needle tip and the collector.

Figure 4. Different morphologies of PCL-gelatin (1:1) microspheres fabricated by electrospray microdripping—solid (A), NF (B), porous NF (C), and hollow NF microspheres (D).

Figure 5. Different compositions of NF microspheres fabricated by electrospray of NF segments— PLGA-gelatin@1:1 (A), PLGA-gelatin@3:1 (B), PLGA NF@10 mg/mL in 0.25 wt % gelatin, (C) and bioactive glass NF@10 mg/mL in 0.25 wt % alginate (D).

Figure 6.

Comparative proliferation of rBMSCs on PCL–gelatin (1:1) solid and NF microspheres. The confocal images show cell cytoskeleton in red because of the staining of F-actin with Alexa Fluor phalloidin 546, nuclei in blue because of DAPI, and the microspheres in the bright field transmitted light. The cell proliferation data shown are mean $\pm$ SD of cell nuclei counted from 15 microspheres per group per time point. * indicates statistically significant difference between the two groups with $p < 0.05$, where p denotes that there is no significant difference between the compared means.

Figure 7. Neural differentiation of CE3 mESC on (A) NF and (B) solid microspheres. Confocal images show neural marker β -III-tubulin in deep red with the secondary antibody labeled with Alexa Fluor 647 and the nuclei with DAPI. The images shown in each case correspond to the frames captured at different z-stack focal planes ($z = 15, 30, 45$, and $60 \mu\text{m}$).

Table 1.

Bulk Densities of Different Morphologies of Microspheres Fabricated by Electrospraying

	bulk density (± 1.0) in mg/mL	
	PCL–gelatin (1:1) microspheres	PLGA–gelatin (1:1) microspheres
solid	458.0 (± 10.0)	436.0 (± 10.0)
NF	11.2	9.2
hollow NF	8.6	ND
porous NF	8.0	6.4