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Composite Electrospun Nanofibers for Influencing Stem Cell Fate

Alessandro Polini, Silvia Scaglione, Rodolfo Quarto, and Dario Pisignano

Abstract

The design of new bioactive materials, provided with "instructive properties" and able to regulate stem cell behavior, is the goal for several research groups involved in tissue engineering. This new function, commonly reserved for growth factors, can lead to the development of a new class of implantable scaffolds, useful for accelerating tissue regeneration in a controlled manner. In this scenario, the likely most versatile and effective tools for the realization of such scaffolds are based on nano- and microtechnology. Here, we show how exploiting the electrostatic spinning (ES) technique for producing a nanofibrillar composite structure, by mimicking topographically the extracellular matrix environment, can influence the fate of human bone marrow mesenchymal stem cells, inducing osteogenic differentiation in the absence of chemical treatments or cellular reprogramming. Basic cues on the choice of the materials and useful experimental instructions for realizing composite nanofibrous scaffolds will be given as well as fundamental tips.

Keywords: Tissue engineering, Regenerative medicine, Cell fate, Stem cells, Cell differentiation, Osteogenic differentiation, Nanofibers, Electrostatic spinning, Electrospinning, Composite materials

1 Introduction

Adult stem cells, including bone marrow mesenchymal stem cells (MSCs), have demonstrated their multipotency leading to the formation of cartilage, bone, muscle, connective tissue, or fat (1). The fine control of the differentiation lineage in vitro is commonly obtained by using defined inductive media. Autologous MSCs are routinely used in conventional tissue engineering ex vivo in combination with biomaterials to create a cell-based scaffold in order to replace or improve the tissue regeneration in vivo (2–4). In addition, endogenous MSCs can locate in cell-free implanted scaffolds through a non-completely understood mechanism, named

homing, contributing to the regeneration of the injured tissue and demonstrating their crucial role in the regenerative process (5).

In all these cases, the host response to the implanted scaffold depends on the cell/material interface that affects MSC function and differentiation. For this reason, the design of smart strategies to finely influence the differentiation of MSCs towards specific lineages with a fully material-driven control (i.e., in the absence of chemical treatments or cellular reprogramming) is a challenge in the development of new performing implantable scaffolds. A very few studies report on the control of the differentiation for MSCs in basal medium condition, exploiting micropatterned fibronectin (6–8), silane- or amino-modified surfaces (9, 10), matrix stiffness (11), and nanotopography (12, 13). Similar results can be obtained with a composite extracellular matrix (ECM)-like structure, realized by electrostatic spinning (ES) technique (14).

Employing polymer solutions of both natural and synthetic polymers, ES is gaining great attention as a simple, low-cost, continuous, high-throughput, and versatile technology for realizing polymer nanofibers due to its capability to produce fibers in the submicron range consistently that is otherwise difficult to achieve by using standard fiber-drawing techniques (15, 16). With smaller and ultimately tunable pore size and higher surface-to-volume ratio than regular fibers, electrospun fibers show very peculiar properties and are effectively applied in several research areas, from optoelectronics to biological applications (17–20). In tissue engineering, electrospun scaffolds aim to topologically mimic the physiological ECM structures that are indeed three-dimensional architectures mainly made by nanofibrous architectures of proteins (21). In this field, the huge versatility of ES, in terms of chemical composition, morphology, and possibility of surface modification of realized fibers, embraces tissue engineering requirements for accomplishing specific cellular requirements (14, 22–24).

This technique is based on the uniaxial stretch of a viscoelastic polymer jet under an intense applied electric field. The experimental setup is extremely simple (Fig. 1): a syringe pump allows to pull out a drop of a polymer solution at the end of a syringe metal needle; a high-voltage supply connected to the needle provides a bias (commonly, in the range 5–50 kV); a metal surface, placed at a distance in the range 5–50 cm and negatively biased, collects solid polymer nanofibers (for modifications to this standard setup, see ref. 20, 25). On the other hand, the spinning physical mechanism is rather complicated. Based on experimental observations and electrohydrodynamic theories, mathematical models have been developed by several groups to describe the ES process (15, 26, 27). From a simplistic point of view, the bias generates a distribution of electrostatic charges on the surface of the droplet at the capillary edge, and when the electric field overcomes a critical value, at which the electrostatic force intensity is larger than the surface tension of the solution,

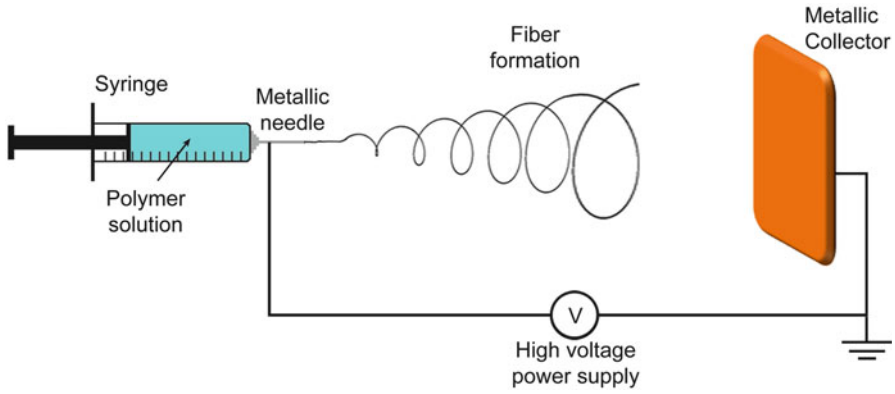

Fig. 1 Schematic drawing of the ES process in a horizontal setup. In the case of a vertical setup, the collector is placed on the hood bench and the needle in a higher position

the droplet becomes more and more unstable and a jet is formed from the needle to the collector. During the needle–collector path, the solvent evaporates, and the resulting polymer fiber is collected onto a substrate. Three different types of processing parameters affect the morphology and diameter of electrospun fibers: (a) the intrinsic properties of the solution, including the type of polymer, its chain conformation, viscosity (or concentration), electrical conductivity, and the solvent polarity and surface tension; (b) the operational conditions, including the strength of applied electric field, the needle–collector distance, and the feeding rate for the polymer solution; and (c) the environmental conditions, such as temperature and relative humidity (28).

In this chapter, we show how the ES technique can be applied for the realization of an ECM-like composite structure, able to influence the differentiation of human bone marrow mesenchymal stem cells (hMSCs) towards the osteogenic lineage in vitro, in the absence of inductive chemical treatments or cellular reprogramming. Specific guidelines and fundamental tips are described in order to overcome typical experimental issues.

2 Materials

2.1 Polymer Solution for ES

1. Organic solvent: Hexafluoroisopropanol (HFIP, >99 %; Carlo Erba), see Note 1.
2. Polymer: Polycaprolactone (PCL) powder (M_w 70,000–90,000; Sigma-Aldrich), see Note 2.
3. Ceramic nanocrystals (NCs: β-tricalcium phosphate, TCP), commercially obtained (Berkeley Advanced Biomaterials) or lab-made by sol–gel method.

4. Oleic acid (>99 %; Sigma-Aldrich).
5. Glass vials and stirring bars.
6. Magnetic stir plate.

2.2 Scaffold Realization

1. Conventional aluminum foil, copper grids with 300 mesh (TAAB Laboratories Equipment), and round borosilicate coverslips (Carlo Erba) as substrate for collecting the fibers, in addition to a 10 × 10 cm^2 metal collector, and carbon tape if needed. See Note 3.
2. Plastic syringes, metal needles (27 gauge), and syringe infusion pump (Harvard Apparatus).
3. High Voltage power supply (EL60R0.6–22, Glassman High Voltage). See Note 4.
4. A chemical fume hood, where the ES setup is placed. The air relative humidity and temperature conditions should be kept constant at about 40 % and 23 °C, respectively.
5. Spin coating apparatus or similar film deposition apparatus for realizing polymer film as reference material for in vitro studies. See Note 5.
6. A vacuum desiccator for samples storage.

2.3 Scaffold Characterization

1. Scanning electron microscopy (SEM) apparatus (Raith 150 system) and an imaging analysis software (WSxM from Nanotech Electronica or ImageJ from NIH).
2. Transmission electron microscopy (TEM) apparatus (Jeol Jem 1011).
3. Fourier-transform infrared (FTIR) spectroscopy system (Spectrum 100, Perkin Elmer). See Note 6.

2.4 Cell Culture

2.4.1 MSC Expansion

1. Human MSCs (Lonza). See Note 7.
2. Basal medium (BM): Mesenchymal Stem Cell Growth Medium (MSCGM, Lonza). Store at 4 °C.
3. Osteogenic medium (OM): Differentiation BulletKit, Lonza. Store at 4 °C.
4. Phosphate-buffered saline (PBS), without magnesium and calcium, pH 7.4 (Euroclone).
5. Trypsin 0.05 %/0.2 % EDTA in PBS without Phenol Red, magnesium, and calcium (Euroclone). Store at −20°C. When ready to use, store at 4 °C.
6. 100-mm round cell culture dishes.
7. Pasteur pipettes.
8. A humidified 5 % CO_2, 37 °C incubator.

2.4.2 Cell Counting

1. 0.4 % (w/v) Trypan Blue solution (Sigma Aldrich).
2. Hemacytometer.
3. PBS (Euroclone).

2.4.3 Cell Adhesion

1. Sodium cacodylate buffer, at pH 7.2: 50 ml of 0.2 M anhydrous sodium cacodylate and 4.2 ml of 0.2 M HCl in a volume of 200 ml of distilled water.
2. Glutaraldehyde 2.5 % in cacodylate buffer. CAUTION: Toxic irritant. Prepare in fume hood and wear appropriate skin and eye protection.
3. 70 % Ethanol. CAUTION: Flammable. Stored in a flammable safe cabinet.

2.4.4 Cell Proliferation and Early Differentiation

1. AlamarBlue (Invitrogen). Store at 4 °C.
2. 15- and 50-ml centrifuge tubes.
3. ALP staining kit (Sigma kit 86-R, Sigma-Aldrich).

2.4.5 RNA Extraction

1. RNAeasy micro kit (Qiagen).
2. Deoxyribonuclease I (Invitrogen).
3. Eppendorf Mastercycler ep Realplex2 (Eppendorf).
4. RealMasterMix SYBR Green (5prime).
5. Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen).
6. Ethanol 70 %.
7. DNase I stock solution.
8. Primers and probe for human RUNX2, Coll I, and BSP were designed as reported in Table 1. GAPDH was used as reference gene.

Table 1
Primer sequences and annealing temperature for real-time RT-PCR

	Primer forward	Primer reverse	T_m (°C)
GAPDH	5′-ACC CAC TCC TCC ACC TTT GA-3′	5′-CTG TTG CTG TAG CCA AAT TCG T-3′	61
RUNX-2	5′-CTT CAT TCG CCT CAC AAA CA-3′	5′-TTG ATG CCA TAG TCC CTC CT-3′	58
Col-I	5′-TTG CTC CCC AGC TGT CTT AT-3′	5′-TCC CCA TCA TCT CCA TTC TT-3′	58
BSP	5′-GAA GAA GAG GAG ACT TCA AAT G-3′	5′-TAT CCC CAG CCT TCT TGG GA-3′	61

3 Methods

3.1 Preparation of the Polymer Solution

1. For polymer nanofibrous scaffolds, prepare a 3.5 % PCL solution (w/w) using HFIP as solvent, by weighing accurately the polymer content in a glass vial. Add a magnetic bar and the required HFIP. Place the vial on a stir plate for a few hours. See Fig. 2 and Note 8.
2. For composite nanofibrous scaffolds, prepare a 3.5 % PCL–2 % NCs–0.05 % oleic acid solution (w/w) using HFIP as solvent, by weighing the polymer and the NCs in a glass vial as first step. Add a magnetic bar, the required oleic acid, and HFIP. Place the vial on a stir plate for 1 week at moderate stirring speed (≈500 rpm, at room temperature). See Fig. 3 and Note 9.

3.2 Scaffold Production

1. Place the substrate (Al foil, TEM copper grids, and/or round borosilicate coverslips) onto the metal collector placed 20 cm far from the metal needle, using carbon tape if needed. See Note 10.

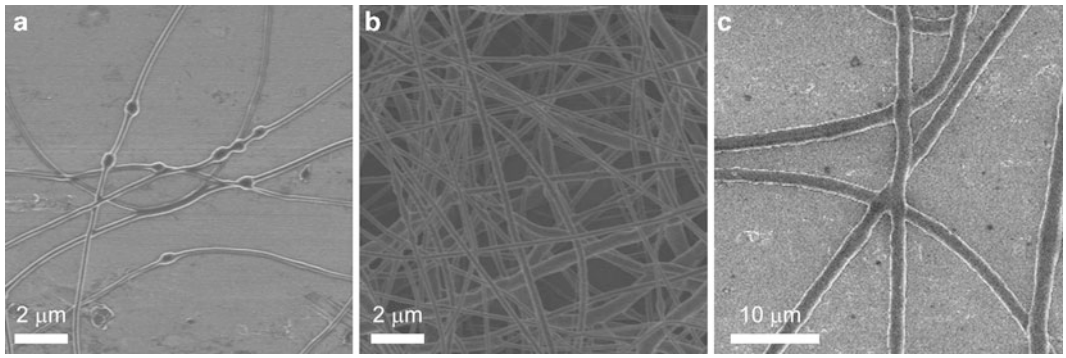

Fig. 2 SEM micrographs of PCL NFs electrospun from 2 % (**a**), 3.5 % (**b**), 7 % (**c**) w/w HFIP solutions

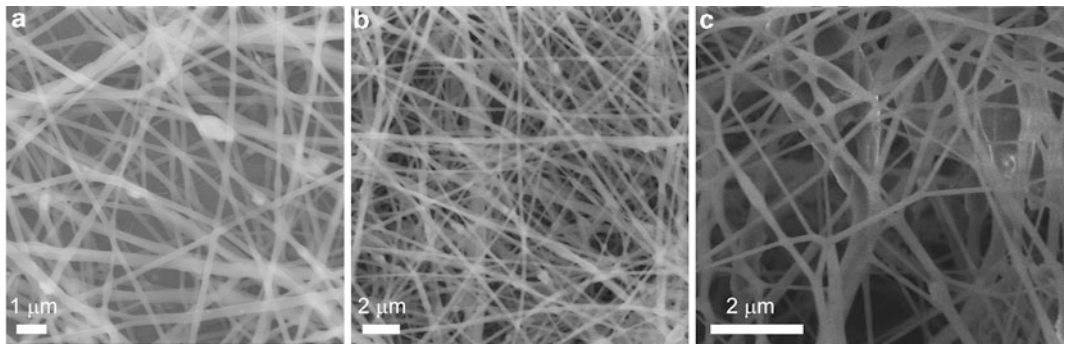

Fig. 3 SEM micrographs of PCL-NC NFs, electrospun from 3.5 to 0.5 % (**a**), 3.5 to 2 % (**b**), 3.5 to 3 % (**c**) w/w HFIP solutions

2. Fill the syringe with the polymer solution and connect it to the syringe infusion pump (0.5 ml/h).
3. Connect the power supply to the needle, increasing the applied voltage gradually up to 4.5 kV.
4. Switch on the pump: At the tip of the needle a cone should appear from the meniscus of the polymer solution, and a white circle should be notable on the collector. See Note 11.
5. Wait for 2 h to obtain a robust sheet of NFs on your substrate. If a TEM grid is used, few seconds are required for having enough NFs to investigate (the presence of too many fibers can dramatically decrease the signal intensity during TEM analysis).
6. Switch off the pump and power supply, decreasing the applied voltage gradually to 0 kV. Store the samples in a vacuum desiccator until use, to allow the complete evaporation of residual solvent molecules (even few hours are enough).
7. For producing reference film materials, use the same ES solution, round glass coverslips, and a spin coating apparatus (5,000 rpm for 60 s).

3.3 Scaffold Characterization

1. SEM (morphology): Cut the Al foil sheet with the NFs into 1 cm × 1 cm pieces and analyze them using an accelerating voltage of 5 kV and an aperture size of 20 mm. Note that if the analysis is difficult due to the low conductivity of the sample, a sputter coating (10 nm) can be employed. Collect several images, using different magnitude, and analyze 100 fibers, at least, by an imaging software for calculating the mean diameter.
2. TEM (morphology): Analyze the TEM grids operating at an accelerating voltage of 100 kV.
3. FTIR (chemical composition): Lift up the NF layer from the Al foil substrates using a razor blade or tweezers, in order to have a 2 cm × 2 cm stand-alone NF sheet. Analyze it in transmission mode, recording spectra in the 450–4,000 cm^{-1} range, with 256 scans at spectral resolution of 2 cm^{-1}. See Note 12.

3.4 In Vitro Studies

MSC isolation and culture steps should be performed in a laminar flow hood using sterile technique and sterile reagents.

3.4.1 MSC Culture and Seeding on Scaffolds

1. Resuspend a pool of five healthy donors of hMSC in MSCGM.
2. Plate cells on round 100-mm tissue culture dishes at a density of $1.7 \times 10^6/cm^2$ and culture them in incubator.
3. After 48 h of incubation, remove nonadherent cells by aspirating away the old media using a sterile Pasteur pipette.
4. Add fresh MSCGM to adherent cells in dishes and feed cells with MSCGM every 2–3 days.

5. Grow cells in vitro to 80 % confluency (10,000 cells/cm^2) before subculturing.
6. Aspirate old media using a sterile Pasteur pipette from the cell culture dishes.
7. Wash cells with sterile PBS. Aspirate PBS.
8. Add 3 ml of 0.5 % trypsin/EDTA to 100-mm round dishes.
9. Incubate at 37 °C for 3 min or longer until about 90 % of the cells detach.
10. Add 5 ml of MSCGM to deactivate trypsin activity; see Note 13.
11. Collect cells and centrifuge at 500 × *g* for 5 min.
12. Aspirate the supernatant, being careful not to disturb cell pellet.
13. Resuspend cell pellet in appropriate amount of MSCGM and break up cell pellet by pipetting up and down.
14. Plate cells onto new 100-mm dishes. Cells are generally subcultured at a dilution ratio of 1:3 to give a density of 3,000–4,000 cells/cm^2.
15. Grow cells in vitro to 80 % confluency (passage P1) and feed cells with MSCGM every 2–3 days.

3.4.2 Cell Counting

1. Trypsinize cells (refer to Section 3.4.1, steps 6–13).
2. Mix thoroughly and draw up 10 μl of cells in media using a micropipette. Mix with 10 μl of Trypan Blue solution (1:2 dilution of cells).
3. Place 20 μl of Trypan Blue-stained cells under a glass coverslip on a hemacytometer.
4. Count the total cells in each corner (one quadrant) of the hemacytometer. Also keep a running tally of the number of Trypan Blue-positive cells (these cells will appear blue).
5. Calculate the average number of cells per quadrant that are Trypan Blue negative. Multiply this number by 2 × 10^4. This number represents the number of cells present per milliliter.

3.4.3 Cell Seeding onto Scaffolds

1. Resuspend cells onto a proper volume of MSCGM, at a density of 0.5–0.8 × 10^6 cells/ml.
2. Pipette 100 μl of cell suspension onto both nanofibrous and film scaffolds. Seed cells also on plastic surface as control, at the same cell density.
3. Check cell seeding under the microscope to make sure that seeding density and distribution are correct. See Note 14.
4. Transfer scaffolds to incubator and let cells attach for 1–2 h.

5. Add either basal (MSCGM) or osteogenic (Differentiation BulletKit) media (hereafter called BM and OM, respectively) onto the scaffolds (both nanofibrous and film scaffolds) so that each 100-mm dish hosting scaffold holds 10 ml media.
6. Culture cell constructs for 2 weeks.

3.4.4 Cell Adhesion onto Scaffolds

At day 7 of culture, stop cell culture onto a sample of nanofibrous and film scaffolds, and process them for SEM analysis.

1. Wash samples in sodium cacodylate buffer for 10 min at 4 °C.
2. Fix in 2.5 % glutaraldehyde buffer for 30 min at 4 °C.
3. Rinse them twice in cacodylate buffer solution at 4 °C for an overall time of 45 min.
4. Dehydrate scaffolds in increasing concentrations of ethanol and air-dry.
5. Sputter-coat with gold samples for 150 s at 60 mA current and below 10^{-1} mbar vacuum. Sputter-coating deposits a conductive metal on the scaffold to enable imaging using the electron beam current.
6. Prepare SEM images of the scaffolds and determine fiber diameter using NIH Image J software available freely at http://rsbweb.nih.gov/ij/.

3.4.5 Cell Proliferation

Cell proliferation assay was performed according to AlamarBlue instructions, every 24 h for a week, in proliferation medium (BM).

1. Add 1/10th volume of AlamarBlue reagent directly to cells in culture medium.
2. Incubate the scaffolds for 3 h in a humidified chamber at 37 °C, protected from direct light.
3. Transfer 250 μl of the incubated solution from each scaffold into a single well of a 96-well plate.
4. Measure the absorbance at 570 nm, using 600 nm as a reference wavelength for normalization.
5. Create a standard curve using samples with known numbers of hMSC to correlate absorbance to cell number.

3.4.6 ALP Activity

ALP enzyme activity of hMSCs, cultured either on NFs or control film, was assessed after 7 days of cell culture in either basal or osteogenic medium. Cell culture on plastic surface was used as control. See Note 15.

1. Use two samples for each experimental condition to assess the ALP activity of the cells using an alkaline phosphatase kit following the manufacturer's instructions.
2. Aspirate media and wash cells with PBS twice.

3. Fix cells with fixative solution for 5 min.
4. Rinse cells briefly with distilled water. Do not allow cells to dry out.
5. Stain cells with alkaline phosphatase staining solution for 30 min at room temperature, according to the ALP staining kit following the manufacturer's instructions.
6. Aspirate staining solution and rinse briefly with water.
7. Take photos under a light microscope of the resulting red, insoluble, granular dye deposit that indicates sites of ALP activity.
8. Convert the images in gray scale (0–255 bit), and select a region of interest (ROI) of 41516 px.
9. Determine the mean intensity value and its standard deviation for each image.

3.4.7 RNA Extraction and RT-PCR

mRNA was isolated and analyzed after 1–2 weeks of culture on plastic or on nanofibrous and film materials, either under BM or OM. See Note 16. RNA extraction was performed following the manufacturer's instructions (RNeasy Micro Kit):

1. Pipette 350 μl of buffer RLT onto the cell-seeded scaffolds, mix well by pipetting up and down, and homogenize the lysate.
2. Add a volume of 70 % (vol/vol) ethanol to the lysate and mix well by pipetting up and down.
3. Take an RNeasy MinElute spin column fitted to a 2 ml collection tube and transfer the whole sample volume onto its membrane.
4. Centrifuge for 15 s at 8,000 × *g* at RT and discard the flow-through.
5. Add 350 μl buffer RW1 to the RNeasy MinElute spin column. Centrifuge for 15 s at 8,000 × *g* at RT and carefully remove the supernatant.
6. Add 10 μl of DNase I stock solution to 70 μl Buffer RDD and mix gently.
7. Add the DNase I Incubator mix (80 μl) directly to the RNeasy MinElute spin column and incubate for 15 min at RT.
8. Add 350 μl of buffer RW1 to the RNeasy MinElute spin column and centrifuge for 15 s at 8,000 × *g* at RT.
9. Place the RNeasy MinElute spin column in a new 2 ml collection tube; add 500 μl of buffer RPE to the spin column, centrifuge for 15 s at 8,000 × *g* at RT, and discard the flow-through.
10. Add 500 μl of 80 % (vol/vol) ethanol onto the RNeasy MinElute spin column, centrifuge for 2 min at 8,000 × *g* at RT, and discard the flow-through.

11. Put the RNeasy MinElute spin column into a new 2 ml collection tube and centrifuge it at full speed for 5 min with an open lid. The opened lid allows the ethanol to flow through the column completely.
12. Put the RNeasy MinElute spin column into a new 1.5 ml collection tube and add 14 μl of RNase-free water directly to the center of the spin column membrane. Centrifuge for 1 min at full speed at RT.
13. Assess the purity of the RNA with a spectrophotometer measuring the 260 nm/280 nm and 260 nm/230 nm optical density (OD) ratios. High RNA quality has a 260 nm/280 nm ratio of >2 and a 260 nm/230 nm ratio of >1.8. As the absorption maximum of proteins is 280 nm, a low 260/280 ratio is an indicator of protein contamination.
14. Treat all mRNA samples with deoxyribonuclease I prior to reverse transcription.
15. Perform first-strand cDNA synthesis using equal amount of RNA samples (2 μl), according to M-MLV RT instructions.
16. Perform real-time PCR reactions, using the Eppendorf Mastercycler ep Realplex2.
17. Analyze by real-time PCR gene expression levels of core binding factor alpha 1 (CBFA1/RUNX-2), collagen type 1 (Col-I), and bone sialoprotein (BSP). Use as housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH).
18. Use RealMasterMix SYBR Green (5prime, Hamburg, Germany) in a total volume of 13 μl for each PCR reaction performed.
19. Assess each sample at least in duplicate.

4 Notes

1. HFIP is an organic solvent with high solubilizing potential for peptides and peptide intermediates, often used as NMR solvent. Its properties, such as electric constant ($\varepsilon = 16.7$, at 25 °C) and boiling point (bp = 59 °C), fulfill the experimental requirements of optimal ES solvents ($\varepsilon > 5$, bp < 100 °C). In ES, it is commonly employed for solubilizing biopolymers as well as synthetic polymers. As alternative, chloroform, dichloromethane, 2,2,2-trifluoroethanol, and formic acid are the most used.
2. Though we focus on the osteoinductive properties of composite PCL-based nanofibers, similar effects could be hopefully achieved by using different biodegradable polymers (natural or synthetic) easily processable by ES. Since the M_w affects

both the rheological and electrical properties of the solution, i.e., viscoelasticity, surface tension, conductivity, and dielectric strength (29), it should be carefully chosen. A low M_w polymer solution generally tends to form beads rather than fibers and a high M_w solution usually gives fibers with larger average diameters (30).

3. An Al foil, placed on the collector, is commonly used as substrate for collecting nanofibers. For in vitro studies, round borosilicate coverslips are preferable as substrate since (a) glass is biologically inert and transparent in the visible range, and (b) it is often difficult to have stand-alone, uniform nanofibrous sheets after the ES process (the fibers are very sticky and easily tend to fold). The diameter of the coverslips should be chosen taking into account the plastic multiwell culture plates used for in vitro studies (e.g., a diameter of 15 mm is suitable for 24-well plates). The TEM copper grids (with or without a carbon film) are necessary for TEM investigation.
4. Two HV power supplies can be occasionally necessary, when the fiber collection is not selective towards the collector. This issue can be due to an electrostatically charged ambient in which the ES apparatus is placed. In this case an HV power supply with positive polarity should be connected to the needle of the syringe and another one with negative polarity to the collector or vice versa (see Fig. 1 for clarity).
5. The choice of the reference material for in vitro studies is important. A polymer film, using the same ES solution, is useful for studying the effect of topography on cell behavior, removing the material chemistry as variable. Here, the use of composite materials requires the realization of fully polymer materials (i.e., without NCs) for studying the effect of the material chemistry. Other reference materials should be bare glass coverslips and plastic treated cell culture plates, with or without a polylysine coating.
6. Other apparatus such as a dynamic mechanical analyzer (i.e., DMA Q800, TA Instruments) and a water contact angle analysis system (i.e., CAM-200 KSV Instruments) can be used for studying the mechanical and wettability properties of nanofibrous mats, respectively. Though these tests offer the possibility to characterize these kinds of materials more in depth, they are not present in all the nanofiber-based studies for tissue engineering applications.
7. In place of performing bone marrow isolation, isolated MSCs are commercially available from various companies.
8. The here reported polymer concentration has been optimized for obtaining almost all of the fibers with a sub-500 nm diameter and very few beads (Fig. 2). The concentration is one of the

most important ES parameters that affects the fiber diameter and the presence/absence of beads. A minimum solution concentration is required. At low solution concentration, both beads and fibers are obtained. As the solution concentration increases, uniform fibers with increased diameters are produced because of the higher viscosity resistance (29). Higher solution concentrations generally lead to fibers with higher diameters.

9. In our experimental approach, firstly the polymer concentration was optimized for obtaining a sub-500 nm fiber diameter, i.e., the same scale of the ECM architectures (21), supposing that the subsequent introduction of NCs has no detrimental effects on the diameter. We then added NCs at the highest concentration (2 % w/w) suitable for electrospinning beadless NFs (higher values lead to the formation of very large clusters of NCs along the fibers, Fig. 3). Moreover, both the introduction of a surfactant (oleic acid), playing as mediator in the interaction between the hydrophilic ceramic and the hydrophobic polymer, and a long stirring time (a week) were found essential for avoiding the bead formation and obtaining a homogeneous composite solution.
10. If a horizontal ES setup is used the use of carbon tape can be necessary. In the vertical setup, the substrates can be just placed onto the metal collector without tape. In terms of the ES process, the two setups lead to identical results.
11. When more than one cone is present, the voltage is probably too high. A high voltage leads sometimes to an increase of the local temperature and an early evaporation of the solvent at the needle edge. In contrast, when a solution drop is continuously formed at the needle tip, the flow rate should be decreased.
12. A baseline and average correction after the spectra collection are usually required for NFs, since the samples can be slightly different in terms of thickness. If a saturated signal is obtained, consider to decrease the ES time in order to have thinner sheets.
13. Commercially available media for MSCs is specifically designed with prescreened FBS to keep cells undifferentiated. MSCGM should be used when expanding cells over multiple passages to prevent spontaneous differentiation. MSCGM consists of a basal media with supplements. Supplements are stored at −20 °C and added to basal media, which is stored at 4 °C. When the supplements are added to the basal media to complete the MSCGM formulation, the media is stored at 4 °C and must be used within a month.
14. Be careful to spread cell solution all over scaffold, but not to let the solution fall off the slide. Important: It is critical that the scaffolds be dried correctly so that the cell solution remains on the scaffold layer. Once the solution falls off, it is extremely

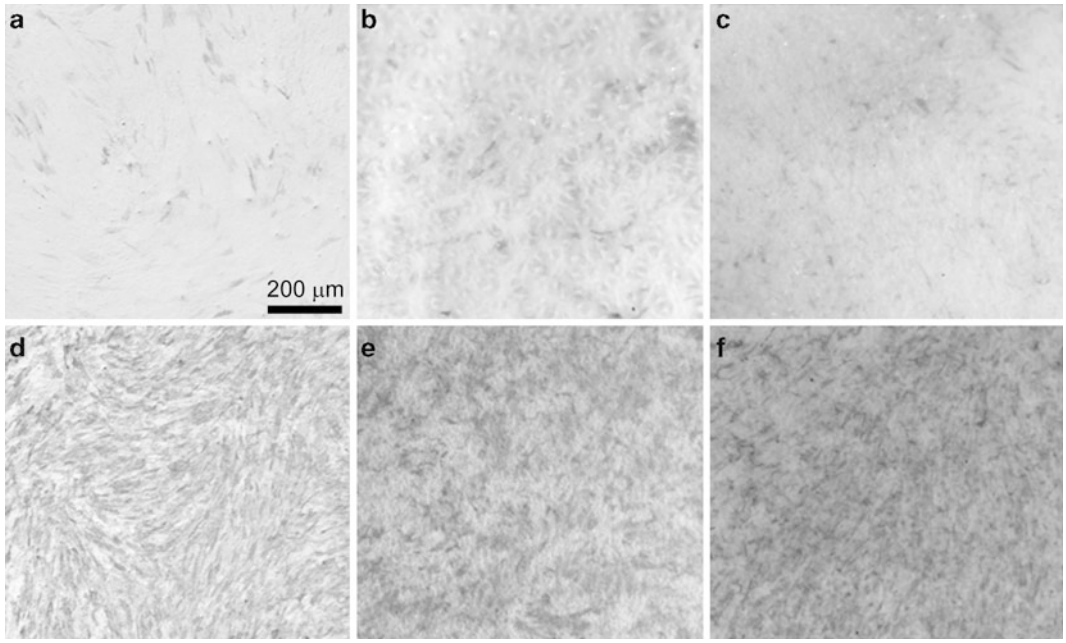

Fig. 4 ALP activity evaluated at day 7 of cellular culture onto PCL NFs (**b**, **e**), PCL-NC NF (**c**, **f**) samples, and control PCL-NC films (**a**, **d**), either in control medium (**a**–**c**) or in osteogenic medium (**d**–**f**)

difficult to put the cells back on and there may be cells that cannot be retrieved from the dish, making seeding uneven.

15. During the osteoblast differentiation, cells initially proliferate up to 7–14 days and then start to produce early differentiation markers and secrete ECM proteins. ALP is an enzyme involved in the pathway resulting in the deposition of minerals on ECM molecules. Therefore, we immuno-histochemically evaluated its activity for hMSCs cultured for 1 week onto samples either in osteogenic or in basal growth medium (Fig. 4).
16. We performed a quantitative analysis of mRNA expression levels, focusing on runt-related transcription factor 2 (Runx-2), BSP, and type I collagen (Col-1) genes, since Runx2 strongly influences the differentiation process of hMSCs into osteogenesis in the early stage, regulating bone development by G protein-coupled signaling pathway and promoting an up-regulation of ALP, osteopontin, osteocalcin, and BSP (31); Col-1 is fundamental for the development of the bone cell phenotype, being correlated to the formation of the ECM; and BSP is a highly sulfated and glycosylated phosphoprotein found in bone matrix, considered one of the late markers of mineralized tissue differentiation (32), and also associated with the capacity for bone formation by MSCs (33). However, this molecular analysis could also be extended to other types of genes, following the same procedural scheme.

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