Journal of Materials Science: Materials in Medicine (2019)30:99
https://doi.org/10.1007/s10856-019-6302-2

TISSUE ENGINEERING CONSTRUCTS AND CELL SUBSTRATES

Rapid Communication

14-3-3ε protein-immobilized PCL-HA electrospun scaffolds with enhanced osteogenicity

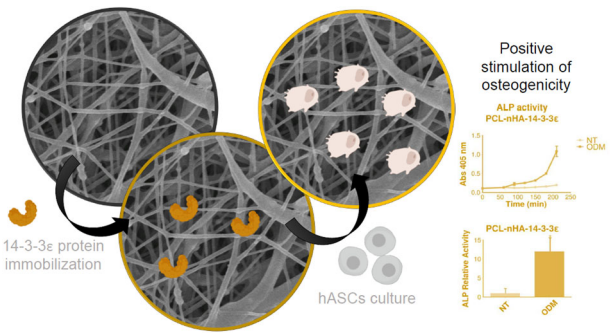
G. Rivero[1] · A. A. Aldana[1,2] · Y. R. Frontini Lopez[3] · L. Liverani[2] · A. R. Boccacini[2] · D. M. Bustos[3,4] · G. A. Abraham[1]

Received: 11 June 2019 / Accepted: 17 August 2019

Abstract

Adipose-derived mesenchymal stem cells (ASCs) accelerate the osteointegration of bone grafts and improve the efficiency in the formation of uniform bone tissue, providing a practical and clinically attractive approach in bone tissue regeneration. In this work, the effect of nanofibrous biomimetic matrices composed of poly(ε-caprolactone) (PCL), nanometric hydroxyapatite (nHA) particles and 14-3-3 protein isoform epsilon on the initial stages of human ASCs (hASCs) osteogenic differentiation was investigated. The cells were characterized by flow cytometry and induction to differentiation to adipogenic and osteogenic lineages. The isolated hASCs were induced to differentiate to osteoblasts over all scaffolds, and adhesion and viability of the hASCs were found to be similar. However, the activity of alkaline phosphatase (ALP) as early osteogenic marker in the PCL-nHA/protein scaffold was four times higher than in PCL-nHA and more than five times than the measured in neat PCL.

Graphical Abstract



1 Introduction

The development of electrospun polymeric and nanocomposite scaffolds for tissue engineering applications is continuously growing [1–3]. The composition is inspired in the hybrid nature of bone: a complex composite made up mostly of organic collagen fibers and hydroxyapatite, a bioactive inorganic calcium phosphate. Biomimetic scaffolds can be designed to direct the organization, growth and/or differentiation of cells in the process of forming functional tissues, providing physical and chemical signals to mimic the bone cell environment [4].

Adipose-derived mesenchymal stem cells (hASCs) are very promising in the field of regenerative medicine due to the many advantages over other types of stem cells like their

These authors contributed equally: G. Rivero, A. A. Aldana

✉ G. A. Abraham
gabraham@fi.mdp.edu.ar

[1] Instituto de Investigaciones en Ciencia y Tecnología de Materiales, INTEMA (UNMdP-CONICET), Mar del Plata, Argentina

[2] Institute of Biomaterials, Department of Materials Science and Engineering, University of Erlangen-Nuremberg, 91058 Erlangen, Germany

[3] Laboratorio de Integración de Señales Celulares, Instituto de Histología y Embriología de Mendoza (IHEM-CONICET-UNCUYO), Mendoza, Argentina

[4] Facultad de Ciencias Exactas y Naturales (UNCuyo), Mendoza, Argentina

Published online: 27 August 2019

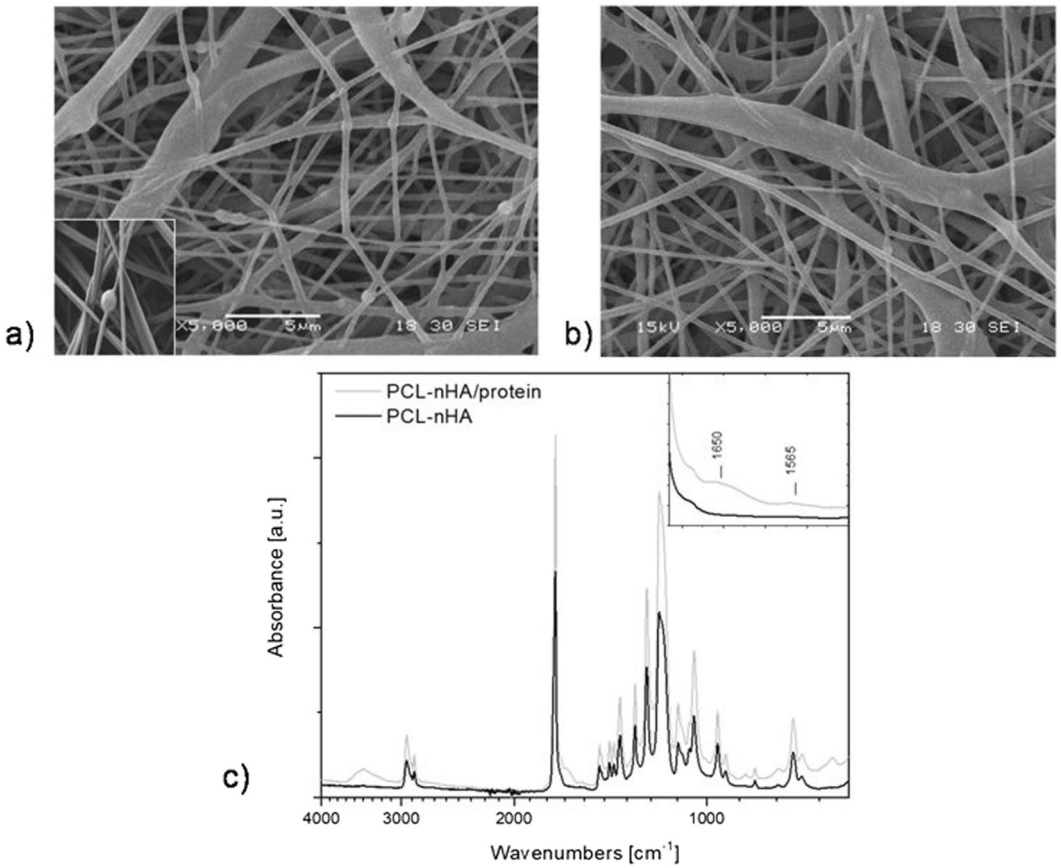


Fig. 1 SEM images of **a** PCL-nHA and **b** surface-modified composite electrospun membranes. **c** ATR-FTIR spectra of PCL-nHA and PCL-nHA/protein

relatively easy isolation, greater performance, yield and proliferation rate and capability of differentiation to multiple lineages [5, 6]. The improvement of bone implants by incorporating hASCs to favor their rapid and efficient osseointegration represents a challenge in this area [7, 8].

The aim of this work was to study the adhesion, viability, proliferation and early stages of osteogenic differentiation capacity of hASCs on nanofibrous matrices composed of poly(ε-caprolactone) (PCL), nanohydroxyapatite (nHA) as bioceramic nanophase and 14-3-3 protein isoform epsilon. This specific protein was recently reported in relation to the development of osteoblasts and related cells [9, 10].

2 Materials and methods

2.1 Scaffold preparation and characterization

Electrospun mats were obtained from 15% w/v poly(ε-caprolactone) solutions (PCL, Aldrich, Germany, Mn ~ 80,000 g mol^{-1}) in acetic acid glacial (VWR, Germany) [11] with 20 wt% of nHA (Aldrich, nanopowder < 200 nm) respect to PCL mass. The dispersion was stirred and ultrasonicated. Electrospinning process was performed at room temperature with environmental relative humidity of 35% in a YFlow® equipment using 15 kV, a distance tip-to-collector of 11 cm and 0.5 mL/h flow rate. Samples were inspected by scanning electron microscopy (SEM, Jeol 6460LV). Fiber diameters were measured with ImageJ software.

2.2 *14-3-3ε* protein recombinant expression on Escherichia coli

14-3-3ε protein was expressed heterologously in *Escherichia coli*. The gene of human 14-3-3ε was cloned in the vector pET24a (Clontech) and purified by using the His6tag in the C-terminal of the protein. Purity and quality were check by SDS-PAGE [12].

2.3 Surface modification of PCL-nHA scaffolds

Membranes were immersed in 5 M sodium hydroxide solution (Sigma Aldrich) for 1 h. Hydrolyzed mats were washed with 0.01 M chloride acid and distilled water to yield fiber surfaces bearing carboxylic groups [13]. 14-3-3

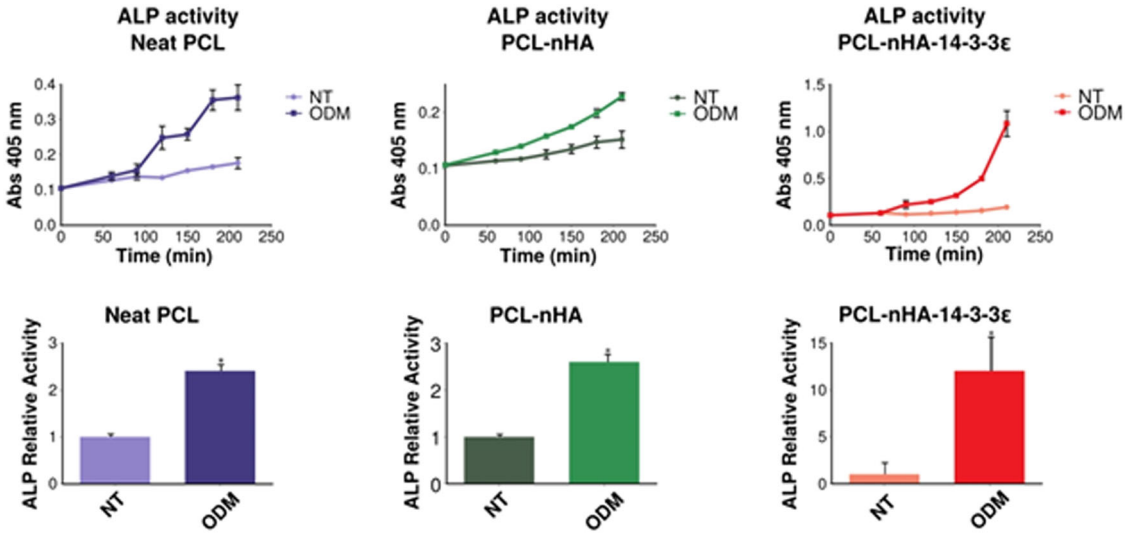


Fig. 2 Enzymatic kinetics of the early biomarker of osteogenesis ALP on non-treated and differentiated hASCs on PCL scaffolds. After three days of growing on either standard DMEM supplemented with 10% fetal bovine serum (NT) or osteogenic differentiation medium (ODM) the activity of ALP was colorimetrically measured by the absorption of its hydrolyzed substrate. Data points represent the mean of three independent experiments in duplicate (±SE). Statistical differences ($p < 0.05$) are indicated as an asterisk. ANOVA/Dunnett's t-tests were performed for each condition and compared to the respective controls

protein was attached to the hydrolyzed-surface of the mats using a coupling reaction with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Sigma) and N-hydroxysulfosuccinimide (NHS, Sigma) [13]. Briefly, mats were rinsed with PBS buffer and then incubated with 500 µL MES buffer (50 mM, 2-(N-morpholino)ethanesulfonic acid, Sigma) for 1 h at room temperature. 500 µL of mixture of EDC (30 mM) and NHS (15 mM) in MES buffer (50 mM) was added to each well. Samples were incubated with protein solution in MES buffer (1 mL, 64 ug/mL) during 3 h at room temperature, washed (PBS ×3) and freeze-dried (Virtis SP Industries, Benchtop 2.0 K) for 48 h.

2.4 Cell culture and ALP activity evaluation

Healthy patients who underwent a dermolipectomy voluntarily donated abdominal subcutaneous adipose tissue after signing an informed consent in accordance with the Declaration of Helsinki (*Universidad Nacional de Cuyo* Bioethics Committee 14594/2014). Material was processed to obtain the hASCs from the stromal vascular fractions [14] and cells characterization was conducted by flow cytometer analysis (CD105, CD90, CD73 positive markers and CD45, CD34, CD11b, CD19, HLA-DR negative markers) and by differentiation to adipogenic and osteogenic lineages [14].

Isolated hASCs were seeded on each scaffold at 40,000 cells/cm^2. Samples were incubated in 6 well plates in standard DMEM supplemented with 10% fetal bovine serum, plus penicillin/streptomycin 100 U/ml (Thermo Fisher Scientific) for three days and adhesion and proliferation were measured by cell counting in Neubauer chamber and/or direct microscopic observation. Viability was measured by alkaline phosphatase (ALP) activity assay. Osteogenic differentiation of hASCs was induced with a drug cocktail containing 10 mM β-glycerophosphate, 0.1 µM dexamethasone and 50 µg/mL 2-phospho-L-ascorbic acid [14]. ALP activity was colorimetrically measured (multiskan FC, Thermo Fisher Scientific) using the absorption of hydrolyzed tetrazolium salt at $\lambda = 405$ nm. Total and steady-state ALP activities were measured through the hydrolysis of *p*-nitrophenylphosphate on fixed and permeated cells (4% *p*-formaldehyde; 0.05% phosphate buffer saline/Tween-20). Results form 6 independent experiments were statistically analyzed using one-way analysis of variance (ANOVA) in GraphPad Prism 5 ($p < 0.05$).

3 Results

Electrospun nanocomposite PCL-nHA scaffolds were successfully obtained using solvents with low toxic potential (Class 3) according to ICH guidelines [11]. PCL-nHA scaffolds showed uniform bead-free nanofibers with 360 ± 160 nm in diameter (Fig. 1a) with certain agglomerations of around 0.5 µm that could correspond to the ceramic phase incorporated inside the fibers, as reported in the inset of Fig. 1a [3]. Morphology of the fibers was not altered by the surface alkaline modification (Fig. 1b). 14-3-3 proteins, particularly the isoform epsilon, have cytokine and osteoinductive activity [10]. The human 14-3-3ε protein was isolated and successfully immobilized onto PCL-nHA scaffolds, as evidenced by ATR-FTIR analysis. Amide I (C=O

stretching) and amide II (N–H bending) signals were detected at 1650 and 1565 cm^{-1}, respectively [10]. The broad peak around 3000–3600 cm^{-1} in PCL-nHA/protein was attributed to the N–H stretching vibration of the protein.

hASCs cells showed a good physiological behavior, morphology and stemness when cultured on all electrospun scaffolds compared to plastic culture plate [13]. The modification of PCL-nHA mats with 14-3-3ε protein enhanced the cell proliferation (from 85% for neat PCL to 105% in PCL-nHA/protein), thus corroborating the protein activity. Moreover, cell adhesion increased from 96% (neat PCL) to 99% (PCL-nHA) and 100% (PCL-nHA/protein).In addition, there was no evidence of death cells in any scaffold.

The early steps of the osteogenic differentiation of hASCs were analyzed by measuring the activity of ALP, a biomarker of the passage from mesenchymal cell to pre-osteoblast/mature osteoblast, which is absent in osteocytes (final lineage) [15]. Cell proliferation on the PCL-nHA and PCL-nHA/protein scaffolds showed more differentiation than that of cells seeded on neat PCL (Fig. 2). The strong effect of the recombinant protein on the hASCs differentiation was evidenced when considering that the PCL-nHA/protein induced four times more ALP activity than PCL-nHA, which was also five times higher than that of PCL mats. Interestingly, the key role of the nanofibrous structure seems relevant, as there was no stimulatory effect on the cell differentiation potential nor in the ALP activity when hASCs were cultured with nHA particles without PCL mats (data not shown). Similar results were observed when analyzing these parameters on hASC growing on PCL-nHA with standard DMEM plus fetal bovine serum. For this reason, the observed ALP activity in PCL-nHA cannot be attributed to the chemical activity of nHA on the cell differentiation or on cellular receptors expressed on the cell surface. The structure of PCL-nHA seems to be an important factor for inducing hASCs differentiation.

4 Conclusion

Human 14-3-3ε protein was isolated and immobilized on composite PCL-based electrospun mats by following a simple methodology. Both the chemical composition and the nanofibrous structure of the scaffolds resulted critical for cell proliferation and differentiation. The positive stimulation of osteogenicity observed in these scaffolds makes them very promising for bone tissue engineering applications. Indeed, synergistic effects of nanotopographic features and biochemical stimuli have started to be investigated in detail to improve tissue regeneration outcomes.

Acknowledgements The authors thank the MINCyT-DAAD 2016 binational cooperation project for partial funding.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Kenry LCT. Nanofiber technology: current status and emerging developments. Progr Polym Sci. 2017;70:1–17.
2. Rivero G, Aldana AA, Abraham GA. Nanocomposite electrospun micro/nanofibers for biomedical applications. In: Grumezescu V, Grumezescu AM, editors. Materials for biomedical engineering: biopolymer Fibers. Amsterdam: Elsevier; 2019. pp. 89–126.
3. He C, Nie W, Feng W. Engineering of biomimetic nanofibrous matrices of drug delivery and tissue engineering. J Mater Chem B. 2014;2:7828–48.
4. Dang M, Saunders L, Niu X, Fan Y, Ma PX. Biomimetic delivery of signals for bone tissue engineering. Bone Res. 2018;6:25.
5. Zuk PA, Zhu M, Mizuno H, Futrell JW, Katz AJ, Benhaim P. et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. Tissue Eng. 2001;7:211–28.
6. Romagnoli C, Brandi ML. Adipose mesenchymal stem cells in the field of bone tissue engineering. World J Stem Cells. 2014;6:144–52.
7. Thomas D, Guthridge M, Woodcock J, Lopez A. 14-3-3 protein signaling in development and growth factor responses. Curr Top Dev Biol. 2005;67:285–303.
8. Kleppe R, Martinez A, Døskeland S, Haavik J. The 14-3-3 proteins in regulation of cellular metabolism. Semin Cell Dev Biol. 2011;22:713–9.
9. Frontini-Lopez YR, Gojanovich AD, Masone D, Bustos D, Uhart M. Adipose-derived mesenchymal stem/stromal cells: from the lab bench to the basic concepts for clinical translation. BIOCELL. 2018;42:67–77.
10. Nefla M, Sudre L, Denat G, Priam S, Andre-Leroux G, Berenbaum F. et al. The pro-inflammatory cytokine 14-3-3ε is a ligand of CD13 in cartilage. J Cell Sci. 2015;128:3250–62.
11. Liverani L, Boccaccini AR. Versatile production of poly(epsilon-caprolactone) fibers by electrospinning using benign solvents. Nanomaterials. 2016;6:75.
12. Uhart M, Iglesias AA, Bustos DM. Structurally constrained residues outside the binding motif are essential in the interaction of 14-3-3 and phosphorylated partner. J Mol Biol. 2011;406:552–7.
13. Guler Z, Sarac AS. Electrochemical impedance and spectroscopy study of the EDC/NHS activation of the carboxyl groups on poly (ε-caprolactone)/poly(m-anthranilic acid) nanofibers. Express Lett. 2016;10:96–110.
14. Gojanovich AD, Masone D, Rodriguez TM, Dewey RA, Delgui LR, Bustos DM. et al. Human adipose-derived mesenchymal stem/stromal cells handling protocols. Lipid droplets and proteins double-staining. Front Cell Dev Biol. 2018;6:33.
15. Miron RJ, Zhang YF. Osteoinduction: a review of old concepts with new standards. J Dent Res. 2012;91:736–44.