



Osteogenic induction of hBMSCs by electrospun scaffolds with dexamethasone release functionality

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ABSTRACT

Electrospun structures were proposed as scaffolds owing to their morphological and structural similarities with the extracellular matrix found in many native tissues. These fibrous structures were also proposed as drug release systems by exploiting the direct dependence of the release rate of a drug on the surface area. An osteogenic differentiation factor, dexamethasone (DEX), was incorporated into electrospun polycaprolactone (PCL) nanofibers at different concentrations (5, 10, 15 and 20 wt.% polymer), in a single-step process. The DEX incorporated into the polymeric carrier is in amorphous state, as determined by DSC, and does not influence the typical nanofibers morphology. *In vitro* drug release studies demonstrated that the dexamethasone release was sustained over a period of 15 days. The bioactivity of the released dexamethasone was assessed by cultivating human bone marrow mesenchymal stem cells (hBMSCs) on 15 wt.% DEX-loaded PCL NFMs, under dexamethasone-absent osteogenic differentiation medium formulation. An increased concentration of alkaline phosphatase and deposition of a mineralized matrix was observed. Phenotypic and genotypic expression of osteoblastic-specific markers corroborates the osteogenic activity of the loaded growth/differentiation factor. Overall data suggests that the electrospun biodegradable nanofibers can be used as carriers for the sustained release of growth/differentiation factors relevant for bone tissue engineering strategies.

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1. Introduction

Drug release systems are used to improve the therapeutic efficacy and safety of drugs, and enhancing the quality of life of patients, by delivering them to the site of action at a rate dictated by the need of the physiological environment [1]. The limitations of the current drug release systems include suboptimal availability, limited effective targeting and potential cytotoxicity resulting from deficient control of the release kinetics. Nanoscale drug release systems can be designed to tune the release kinetics, to regulate distribution and to minimize toxic side effects, thereby enhancing the therapeutic efficacy of a given drug. Drug release with polymer nanofibers is based on the principle that the dissolution rate of a drug increases with increased surface area of both the drug and

the corresponding carrier [2]. Unlike common encapsulation systems involving some complex preparation process [3], electrospinning allows the convenient incorporation of therapeutic/bioactive compounds into the carrier polymer nanofibers in a single-step process [4]. Indeed, it was observed that enzymes and antibiotics encapsulated in electrospun nanofibers preserved higher catalytic activity than the ones incorporated in solvent casting membranes [5,6].

Electrospinning is considered a promising and versatile processing technique, since several polymeric systems [7,8], proteins [9,10] and even living cells [11] can be applied to develop functionally active nanofibrous structures. Recently, electrospun nanofibers were used as a carrier for both hydrophobic and hydrophilic drugs [6,12–14], where the drug release profile can be controlled by the modulation of scaffold morphology, porosity, and composition. In general, the incorporation of bioactive materials (i.e. proteins and growth factors) within electrospun fibers is achieved by “two-phase” electrospinning, where an aqueous solution of the biological molecule is mixed with an organic polymeric solution to form a biphasic suspension [10,15–17], resulting in the encapsulation of

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aqueous reservoirs within the polymer fibers. In another possible alternative, two or more components can be co-electrospun through coaxial capillary channels and integrated into the core–shell biphasic fibrous structure [8]. That strategy leads to difficulties in controlling the kinetics of release of those bioactive molecules, including the burst release effect [18–20]. So far, the research has been directed to obtain the controlled release of anti-inflammatory agents, antibiotics and anticancer agents [21]. The controlled release of growth and differentiation factors from electrospun meshes was not previously explored.

Functional and structural engineering of musculoskeletal tissue, using a tissue engineering approach, may involve the use of appropriated cells cultured with specific growth factors after seeding in biomaterial scaffolds. Controlled release of therapeutic/bioactive molecules from biodegradable scaffolds will enhance the efficacy of tissue engineering approaches [1]. In fact, polymeric biomaterials are frequently used as release systems, since they can readily deliver growth factors at the desired site either by direct protein release approaches or indirectly by gene therapy or by cell transplantation [22]. Consequently, herein we propose the development of electrospun biodegradable nanofibers as a release system of an established osteogenic differentiation factor – dexamethasone – of mesenchymal stem cells (MSCs). Dexamethasone (DEX) is a synthetic glucocorticoid, which has been shown to stimulate MSCs proliferation and to support osteogenic lineage differentiation *in vitro*, together with β -glycerophosphate and ascorbic acid, in defined concentrations [23,24]. β -glycerophosphate, the organic phosphate source, plays an important role in the mineralization process and in modulating osteoblast activities, such as the alkaline phosphatase (ALP) activity and in the expression of osteocalcin. Moreover, ascorbic acid is also essential for increasing cell viability and to stimulate the production of Collagen type I by osteogenic cells. Therefore, the *in vitro* osteogenesis of MSCs might be more successfully established if the culturing substrate/scaffold combines at its surface the adequate physical (i.e. ECM-like nanofibers) and chemical (i.e. growth/differentiation factors) stimulus. Ultimately, we aim at improving the efficacy and reduce the total time required to regenerate a functional bone tissue.

Other authors in the literature reported on electrospun silk fibroin composite fiber scaffolds, containing bone morphogenetic protein-2 (BMP-2) and/or nanoparticles of hydroxyapatite (nHAP), and cultured them with human bone marrow-derived mesenchymal stem cells (hBMSCs) [25]. The results demonstrated that those scaffolds, not only support growth and osteogenic differentiation of hBMSCs, but significantly enhance calcium deposition and mRNA transcript levels of bone-related genes. Recently, in a similar work [26], recombinant human bone morphogenetic protein-2 (rhBMP-2) was loaded into poly(D,L-lactide-co-glycolide)/hydroxyapatite (PLGA/HAp) composite electrospun fibrous scaffolds. Cell culture experiments with BMSCs showed that the encapsulation of HAp could enhance cell attachment to scaffolds and lower cytotoxicity effect. Both works described electrospun fibrous as release system incorporating a growth/differentiation factor with known clinical potential (FDA approved) for bone and cartilage repair [27]. However, the required cocktail of BMPs able to induce a controlled osteogenic differentiation of mesenchymal stem cells is still to be defined.

2. Materials and methods

2.1. Production of nanofiber meshes loaded with dexamethasone

A polymeric solution of poly(ϵ -caprolactone) (PCL) (TONE™, Union Carbide Chemicals and Plastics Division, New Jersey) 17% (w/v) was prepared using a mixture of chloroform and dimethylformamide (7:3), as described elsewhere [28]. Different

quantities (5, 10, 15 and 20 wt.% polymer) of dexamethasone (Sigma–Aldrich, Germany) were added to the polymeric solution previously prepared, and let to stir until complete dissolution was achieved (approximately during 20 min). A tension of 9.5 kV, a current of 3.32 μ A, a needle tip-to-ground collector distance of 20 cm and a flow rate of 1 mL/h were defined as optimized processing conditions for the specific material being processed.

2.2. Characterization of dexamethasone-loaded PCL nanofiber meshes

DEX-loaded electrospun PCL nanofiber meshes were gold sputter-coated (model SC502, Fisons Instruments, England) for 2 min at 15 mA. Samples were analyzed using a Scanning Electron Microscope (model S360, Leica Cambridge, England). Micrographs were recorded at 15 kV with magnifications ranging from 100 to 5000 times.

Fourier Transform Infra-Red Spectroscopy (FTIR) analysis was performed with the DEX-loaded electrospun PCL nanofiber meshes. The samples were powdered, mixed with KBr, and processed into pellets. FTIR spectra were recorded at 48 scans with a resolution of 2 cm^{-1} (Shimadzu – IR Prestige 21)

Differential Scanning Calorimetry (DSC) experiments were carried out using a DSC Q100 equipment (TA Instruments – ELNOR). The experiments were conducted under a nitrogen atmosphere on samples (5–10 mg) packed in aluminium capsules. The samples were heated in two stages at a constant heating rate of 10 $^{\circ}\text{C}/\text{min}$ from room temperature up to 300 $^{\circ}\text{C}$. The samples were kept at this temperature for a period of 2 min and cooled at the same rate to the initial temperature.

2.3. Dexamethasone kinetic release studies

DEX-loaded PCL nanofiber meshes (with 4 cm^2) were weighted and incubated at 37 $^{\circ}\text{C}$ in 30 mL of phosphate buffer solution stirred at 60 rpm. Aliquots of 3 mL were retrieved in predetermined time intervals and the same volume of fresh medium was added to the suspension. The samples were analyzed by UV–Vis spectroscopy at 242 nm (Shimadzu UV 1601). The DEX concentration of each sample was calculated using a standard curve (concentrations ranging from 0.0 to 72 $\mu\text{g}/\text{mL}$), relating the quantity of DEX with the intensity of light absorbance. The results presented are an average of three measurements. Calculations of the amount of drug released took into account the replacement of aliquots with fresh medium. Unloaded control PCL nanofiber meshes (PCL NFMs) were considered as blanks in the performed quantification.

2.4. Expansion, seeding and osteogenic differentiation of human bone marrow mesenchymal stem cells

Human bone marrow mesenchymal stem cells (hBMSCs) (Biopredic International, France) were isolated and characterized according to the method established by Delorme and Charbord [29]. hBMSCs were expanded in basal medium consisting of MEM alpha medium (α -MEM; Gibco, GB) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biochrom AG, Germany) and 1% antibiotic/antimycotic solution (final concentration of penicillin 100 units/mL and streptomycin 100 $\mu\text{g}/\text{mL}$; Gibco, GB). Cells were cultured at 37 $^{\circ}\text{C}$ in an atmosphere of 5% CO_2 .

Before the *in vitro* studies, PCL nanofiber meshes loaded with 15 wt.% dexamethasone were cut in samples with areas of approximately 1 cm^2 and sterilized by UV irradiation during 1 h on each side of the mesh. Confluent hBMSCs at passage 4 were harvested for seeding onto the DEX-loaded PCL NFMs at a density of 1.0×10^5 cells/ cm^2 of the nanofiber mesh. Unloaded PCL nanofiber meshes were used as controls. The DEX-loaded PCL NFMs-hBMSCs constructs were cultured under static conditions, in basal and dexamethasone-absent osteogenic differentiation media (basal medium supplemented with 50 $\mu\text{g}/\text{mL}$ ascorbic acid and 10 mM β -glycerophosphate). The control PCL NFMs-hBMSCs constructs were also cultured under static condition, in basal and standard osteogenic differentiation media (basal medium supplemented with 50 $\mu\text{g}/\text{mL}$ ascorbic acid, 10 mM β -glycerophosphate and 10^{-7} M dexamethasone). The constructs were retrieved at different culture times: 7, 14 and 21 days.

2.5. Cell morphology and distribution, and chemistry of deposited matrix

For scanning electron microscopy (SEM) observation, the samples were fixed with 2.5% Glutaraldehyde (Sigma; USA) in a Phosphate Buffer Saline solution (Sigma; USA) during 1 h at 4 $^{\circ}\text{C}$. The samples were further dehydrated through increasing series of ethanol concentrations and let to dry overnight. Previous to the analysis by SEM (model S360, Leica Cambridge, England) equipped with an energy dispersive spectrometer (EDS; link-eXL-II), the samples were gold or carbon sputter-coated (sputter coater model SC502, Fisons Instruments, England).

2.6. Cell viability and proliferation assessment

Cell viability for each culturing time was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA). This assay is based on the bioreduction of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium [MTS], into a water-soluble

brown formazan product. The absorbance was measured at 490 nm in a microplate reader (Synergie HT, Bio-Tek, USA), being related with the quantity of formazan product and directly proportional to the number of living cells in the constructs. Three samples of each nanofiber mesh per time point were characterized.

Cell proliferation was quantified by the total amount of double-stranded DNA, along the culturing time. Quantification was performed using the Quant-iT™ PicoGreen dsDNA Assay Kit (Invitrogen™, Molecular Probes™, Oregon, USA), according to the instructions of the manufacturer. Briefly, cells in the construct were lysed by osmotic and thermal shock and the supernatant used for the DNA quantification assay. The fluorescence of the dye was measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm, in a microplate reader (Synergie HT, Bio-Tek, USA). Triplicates were made for each sample and per culturing time. The DNA concentration for each sample was calculated using a standard curve (DNA concentration ranging from 0.0 to 1.5 µg/mL) relating quantity of DNA and fluorescence intensity.

2.7. Alkaline phosphatase quantification

The concentration of alkaline phosphatase (ALP) was determined for all time culture periods, using the lysates used for DNA quantification. Briefly, the ALP quantity was assessed using the *p*-nitrophenol assay, in which the colourless nitrophenyl phosphate disodium salt (pNPP; Sigma, USA) is hydrolysed by the ALP (Sigma, USA) at pH 10.5 and temperature of 37 °C to form yellow free *p*-nitrophenol. The reaction was stopped by addition of 2 M NaOH (Panreac Quimica, Spain) and the absorbance read at 405 nm in a microplate reader (Bio-Tek, Synergie HT, USA). Standards were prepared with 10 µmol/mL *p*-nitrophenol (pNP; Sigma, USA) solution, to obtain a standard curve ranging from 0 to 0.250 µmol/mL. Triplicates of each sample and standard were made, and the ALP concentrations read off from the standard curve.

2.8. Alizarin red staining

After culture, the DEX-loaded nanofiber meshes-hBMSCs constructs were fixed in 10% formalin solution neutral buffer (Sigma–Aldrich, Germany) for 30 min and maintained in phosphate buffer saline (PBS) until further use. The constructs were further stained with a 2% Alizarin Red solution (Merk, Germany) in distilled water for 5 min, and finally washed with distilled water. Stained constructs were observed under an optical microscope (BX61, Olympus Corporation, Germany) and images captured by a digital camera (DP70, Olympus Corporation, Germany).

2.9. Immunodetection of bone-specific proteins

Immunocytochemistry was performed following the streptavidin–biotin–peroxidase complex approach (R.T.U. Vectastain® Universal Elite® ABC kit; Vector Laboratories Inc., Burlingame, CA), using a rabbit polyclonal antibody against osteopontin (Abcam Ltd., Cambridge, UK; dilution 1:1500), a mouse monoclonal antibody against osteocalcin (clone OC4-30, Abcam Ltd., Cambridge, UK; dilution 1:100) and a rabbit polyclonal antibody against bone sialoprotein II (Chemicon® International Inc., Germany; dilution 1:2500). Prior to the immunocytochemistry procedure, constructs were fixed in 10% formalin solution neutral buffer (Sigma–Aldrich, Germany) for 30 min and maintained in phosphate buffer saline (PBS) until further use. The constructs were treated with 0.3% hydrogen peroxide in methanol during 30 min to inactivate the endogenous peroxidases. After washing with PBS, the constructs were blocked with 2.5% normal horse serum for 20 min at room temperature to avoid unspecific reactions. Primary antibodies were incubated overnight at 4 °C. Negative controls were set in the absence of primary antibodies incubation. After washing in PBS, the samples were incubated for 30 min with biotinylated secondary antibody anti-rabbit/mouse IgG, followed by incubation with streptavidin–peroxidase complex (Elite ABC Reagent). The immune reaction was visualized using DAB as a chromogen (DAB Substrate Reagent from Peroxidase Substrate Kit; Vector Laboratories Inc, Burlingame, CA). The constructs were observed under an optical microscope (BX61, Olympus Corporation, Germany) and images captured by a digital camera (DP70, Olympus Corporation, Germany).

2.10. RNA isolation and real-time quantitative polymerase chain reaction (qPCR)

Total RNA from the constructs was extracted using the Trizol® (Invitrogen, Life Technologies Inc., UK) method according to the manufacturer's directions. Briefly, at each culturing time the constructs were washed with PBS, immersed in Trizol and storage at –80 °C until further use. Proteins were removed with chloroform extraction, and the RNA pellets were washed once with isopropyl alcohol and once with 70% ethanol. The total RNA pellets were reconstituted in RNase free water (Gibco, Invitrogen, UK).

Reverse transcriptase (RT)-PCR was performed according to the protocol from iScript™ cDNA synthesis kit (BioRad, Hercules, CA, USA). Briefly, a reaction mixture consisting of 1× iScript Reaction Mix, 1 µL iScript Reverse Transcriptase, RNA template (300 ng total RNA) and nuclease-free water was prepared, in 20 µL of total volume. The single-strand cDNA synthesis occurred by incubating the complete

reaction mixture 5 min at 25 °C, followed by 30 min at 42 °C and terminated by an incubation at 85 °C for 5 min.

Amplification of the target cDNA for real-time PCR quantification was performed according to manufacturer, using 2 µL RT cDNA products, 1 µM each primer (bone-specific primer sets listed in Table 1), 1× iQ SYBR Green Supermix (BioRad, Hercules, CA, USA) and nuclease-free water, in a final volume of 25 µL. Forty-four cycles of denaturation (95 °C, 10 s), annealing (temperature dependent on the gene, 30 s) and extension (72 °C, 30 s) were carried out in the gradient thermocycler MiniOpticon real-time PCR detection system (BioRad, Hercules, CA, USA) for all genes. The transcripts expression data were normalized to the housekeeping gene glyceraldehyde-3-phosphate-dehydrogenase (*GAPDH*) and the relative quantification calculated by the $\Delta\Delta C_T$ method.

2.11. Statistical analysis

Statistical analysis was performed using the SPSS statistic software (Release 15.0.0 for Windows). Firstly, a Shapiro–Wilk test was used to ascertain about the data normality. The results indicated that nonparametric tests should be used for all comparisons. A Kruskal–Wallis test followed by Turkey's HSD test were performed to analyze the effect of the DEX-loaded electrospun nanofibrous meshes on the hBMSCs' viability and proliferation, ALP quantification, and osteogenic genotype. *P* values lower than 0.01 were considered statistically significant.

3. Results

3.1. Physicochemical properties of the dexamethasone-loaded electrospun PCL nanofiber meshes

In the present work, a glucocorticoid drug – dexamethasone – was dissolved at different concentrations (5, 10, 15 and 20 wt.% polymer) in the polymeric solution to be electrospun. A mesh-like structure composed by randomly distributed fibers with diameters ranging from 150 nm–1.6 µm (Fig. 1B–E) was produced. The incorporation of dexamethasone at concentrations ranging from 5 to 15 wt.% (Fig. 1B–D) shows the appearance of thinner fibers in larger number than in the control nanofiber meshes (Fig. 1A). However, those differences in morphology are probably insufficient to induce any specific cell response. This observation was expected since the solvent is able to dissolve simultaneously the polymer and the incorporated drug. In this way, the drug is very well dispersed in the bulk polymeric matrix of the electrospun nanofibers without inducing significant morphological variation of the electrospun fibers. Relevant morphological alteration, namely an increment of nanofibers diameter, was observed only in the electrospun meshes with an incorporation of 20 wt.% DEX (Fig. 1E).

To confirm the presence of dexamethasone incorporated in the electrospun PCL nanofibers an FTIR analysis was performed. Fig. 2 shows the spectra of PCL nanofibers, raw dexamethasone and also of the different DEX-loaded electrospun PCL nanofiber meshes. The characteristic peaks of dexamethasone at 900 and 1650 cm⁻¹ are observed in the nanofiber meshes prepared with the different

Table 1
Primers list of osteogenic markers.

Gene		Primer sequences (5'-3')	<i>T_m</i> (°C)
ALP	Sense	CTCCTCGGAAGACACTCTG	60.0
	Antisense	AGACTGCGCCTGGTAGTTG	
OP	Sense	GGGACAACCTGGAGTGAAAA	58.4
	Antisense	CCCACAGACCCTTCCAAGTA	
BSP	Sense	CAACAGCAGACAGGCAGAAAAAC	59.9
	Antisense	CCTCGTATTCAACGGTGGTG	
OC	Sense	CTGAGAGGAGCAGAAGCTGG	61.4
	Antisense	GGCAGCGAGGTAGTGAAGAG	
Runx2	Sense	TTCCAGACCAGCAGCACTC	58.1
	Antisense	CAGCGTCAACACCATCATTC	
Osterix	Sense	CCCTTTACAAGCACTAATGG	57.1
	Antisense	ACACTGGGCAGACAGCTCAG	
GAPDH	Sense	ACAGTCAGCCGATCTTCTT	58.4
	Antisense	GACAAGCTTCCCCTTCTCAG	

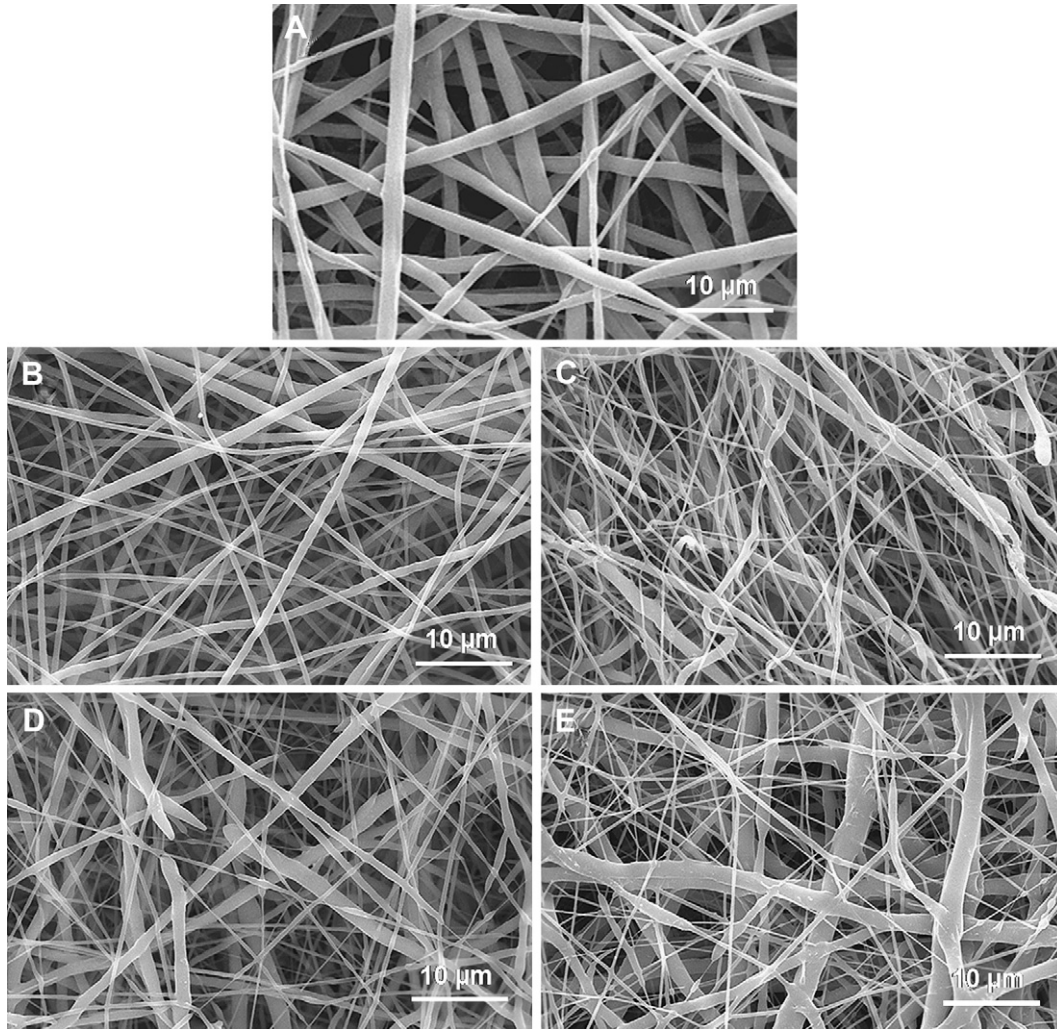


Fig. 1. Morphological characterization of a typical electrospun PCL nanofiber mesh (A) and the electrospun nanofiber meshes incorporating different concentrations of dexamethasone: 5 wt.% (B), 10 wt.% (C), 15 wt.% (D) and 20 wt.% (E).

DEX concentrations, which confirms the presence of the glucocorticoid in the polymeric matrix.

Thermal analysis of the DEX-loaded nanofiber meshes was carried out to ascertain about the crystalline state of the incorporated dexamethasone. Fig. 3A shows the DSC thermograms of

PCL nanofiber meshes and raw dexamethasone. In this graph is possible to observe the melting peaks of the polymer at 56.9 °C and of dexamethasone at 262.2 °C. However, the characteristic endothermic peak of dexamethasone is not present in the DEX-loaded nanofiber meshes (Fig. 3B). These thermal properties of

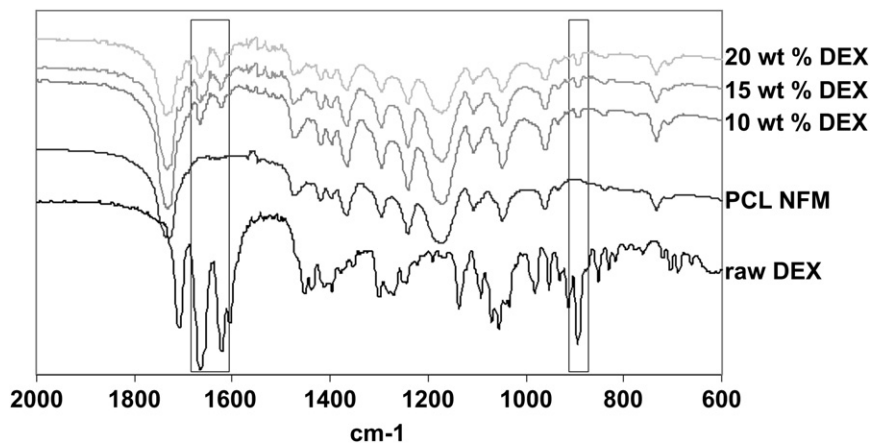


Fig. 2. FTIR spectra of raw dexamethasone, control PCL NFMs, DEX-loaded PCL NFMs at 10 wt.%, 15 wt.% and 20 wt.%.

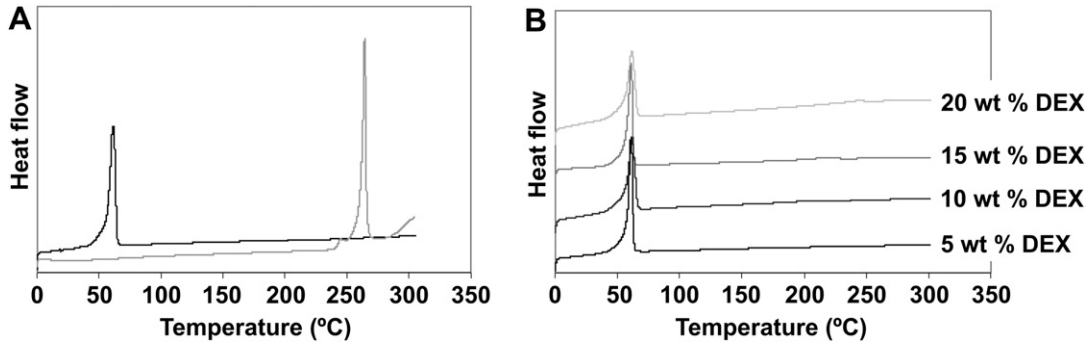


Fig. 3. DSC thermogram of control PCL NFMs (black line) and raw dexamethasone (grey line) (A), and DEX-loaded PCL nanofibers at 5 wt.%, 10 wt.%, 15 wt.% and 20 wt.% (B).

DEX-loaded PCL nanofiber meshes suggest that dexamethasone, precipitated from the polymeric/organic solution during the electrospinning processing, is not in its crystalline state when incorporated in the nanofiber meshes. This is a typical observation, since the mobility of the DEX inside the fibers is highly hindered.

3.2. Release kinetics of dexamethasone from the electrospun PCL nanofiber meshes

The release profile of dexamethasone from the different DEX-loaded PCL NFMs was followed during 21 days, in accordance with the culture time usually needed to observe a complete osteogenic

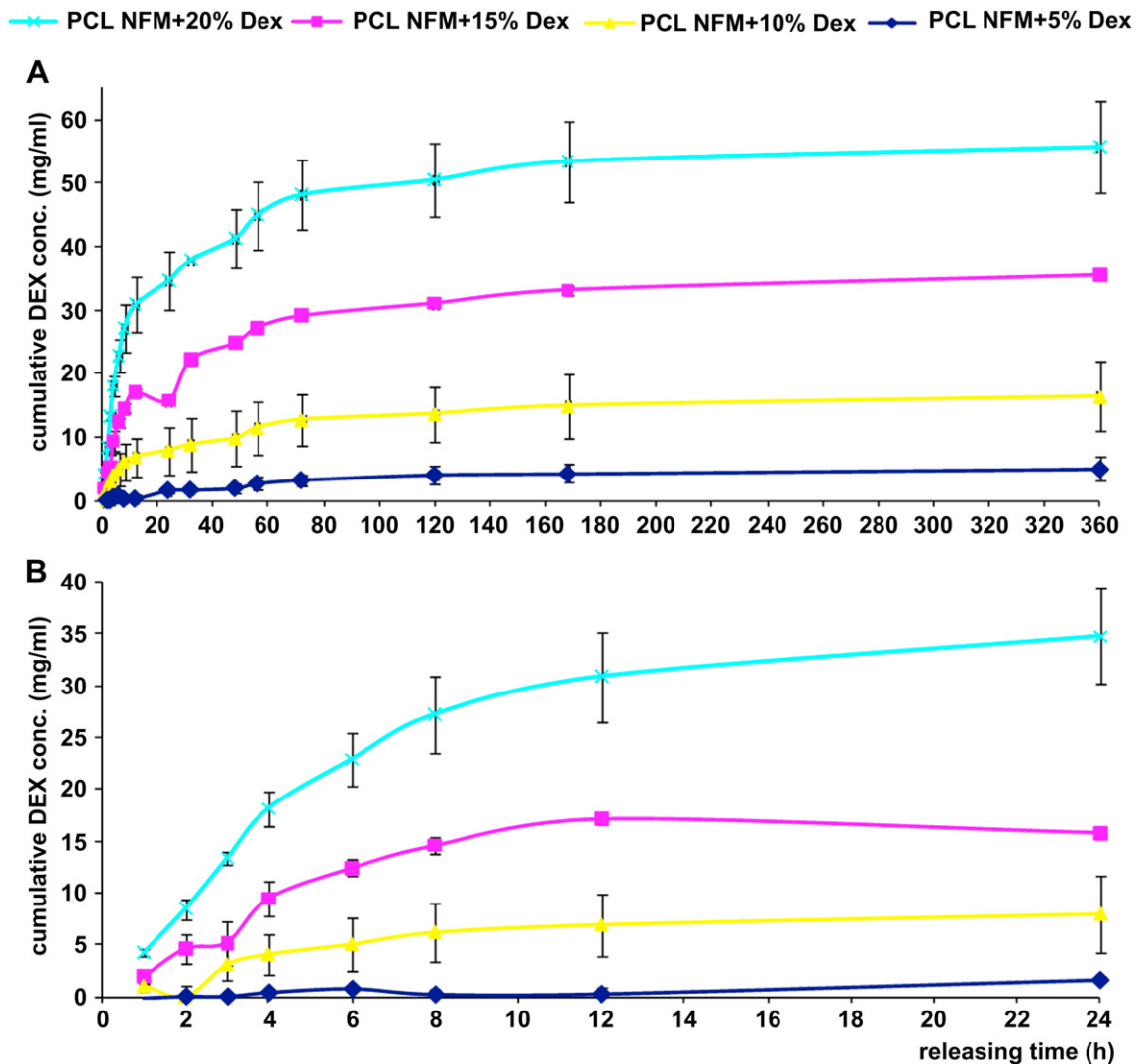


Fig. 4. Release profile of the different dexamethasone concentrations incorporated into the electrospun PCL nanofiber meshes.

differentiation of MSCs *in vitro*. As depicted in Fig. 4, a significant initial release of dexamethasone – burst release – was observed in the case of PCL NFMs incorporating above 5 wt.% of the growth/differentiation factor. It was noticed that a higher amount of drug lead to a faster release rate. After 100 h, a slow sustained release of dexamethasone was observed for all systems. Considering the release profiles of the different DEX-loaded PCL NFMs and the dexamethasone concentrations achieved, the 15 wt.% DEX-loaded PCL NFMs was chosen for further *in vitro* biological assays.

3.3. Biological activity of the dexamethasone released from the electrospun nanofiber meshes

To ascertain about the biological activity, and inherent structural integrity, of the released dexamethasone from the electrospun PCL NFMs, human bone marrow mesenchymal stem cells (hBMSCs) were cultured on 15 wt.% DEX-loaded PCL NFMs. From the MTS assay data (Fig. 5) it was possible to observe that, until the 14th day of culture, the different culture conditions did not induce significant changes over hBMSCs viability ($p > 0.01$). However, at day 21, hBMSCs cultured on DEX-loaded NFMs on dexamethasone-absent osteogenic differentiation medium (*NFM+DEX_Osteo*), on DEX-loaded NFMs (*NFM+DEX_Basal*) and on control PCL NFMs (*NFM_Basal*) on basal medium showed significant lower viability than those cultured on standard osteogenic differentiation medium (*NFM_Osteo*) ($p < 0.01$). Moreover, for this culturing time, DEX-loaded NFMs cultured on basal (*NFM+DEX_Basal*) and dexamethasone-absent osteogenic differentiation media (*NFM+DEX_Osteo*) presented significantly higher hBMSCs' viability than those cultured on control PCL NFMs under basal conditions (*NFM_Basal*) ($p < 0.01$). Complementary to the analysis of hBMSCs' viability along the course of the experiment, the proliferation or replication rate of those cells was also quantified based on the DNA content (Fig. 6). DEX-loaded NFMs constructs cultured on dexamethasone-absent osteogenic differentiation medium (*NFM+DEX_Osteo*) present significant lower proliferation capability than hBMSCs cultured under all the other

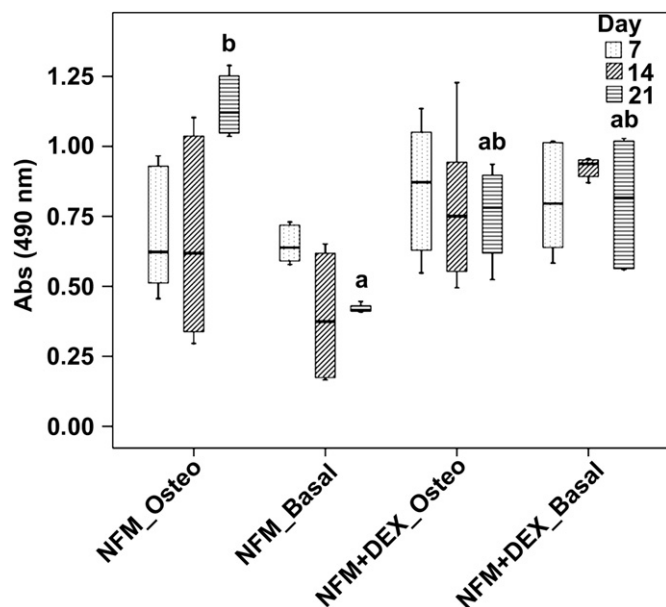


Fig. 5. Box plot of the hBMSCs viability (MTS assay) cultured on *NFM_Osteo*, *NFM_Basal*, *NFM+DEX_Osteo* and *NFM+DEX_Basal*, after 7, 14 and 21 days. Data were analyzed by nonparametric way of a Kruskal–Wallis test followed by Tukey's HSD test: a, denotes significant differences compared to *NFM_Osteo*; b, denotes significant differences compared to *NFM_Basal*.

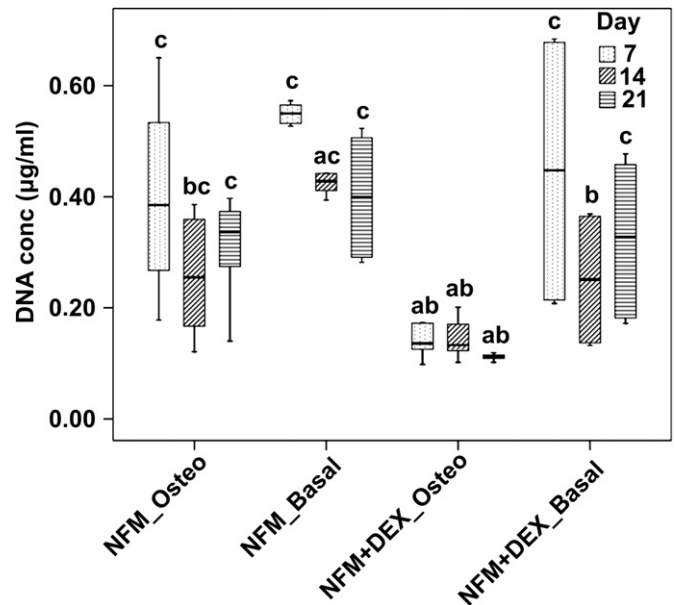


Fig. 6. Box plot of the hBMSCs proliferation (DNA concentration) cultured on *NFM_Osteo*, *NFM_Basal*, *NFM+DEX_Osteo* and *NFM+DEX_Basal*, after 7, 14 and 21 days. Data were analyzed by nonparametric way of a Kruskal–Wallis test followed by Tukey's HSD test: a denotes significant differences compared to *NFM_Osteo*; b denotes significant differences compared to *NFM_Basal*; c denotes significant differences compared to *NFM+DEX_Osteo*.

conditions ($p < 0.01$). Conversely, control PCL NFMs cultured under basal medium (*NFM_Basal*) demonstrated significantly higher DNA concentration than all the other substrates and corresponding culture medium ($p < 0.01$), just for the 14th day.

Despite the above described proliferative cell population cultured on the DEX-loaded PCL NFMs under basal (*NFM+DEX_Basal*) or dexamethasone-absent osteogenic differentiation media

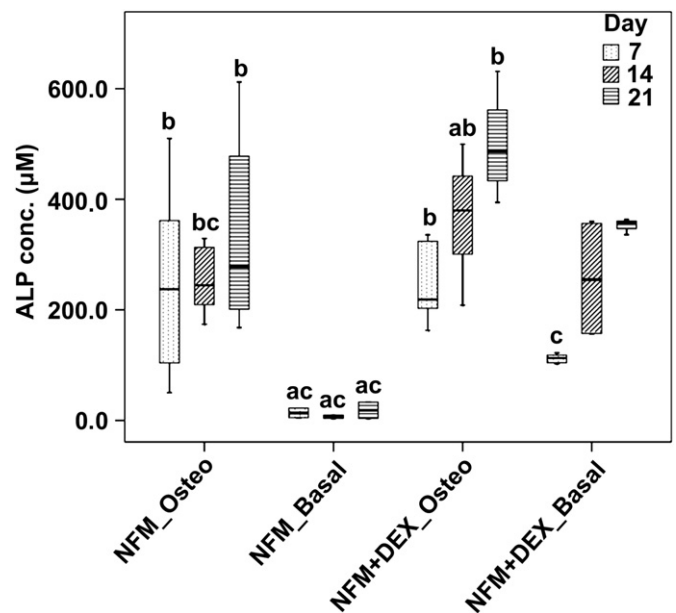


Fig. 7. Box plot of the alkaline phosphatase (ALP) concentration produced by hBMSCs cultured on *NFM_Osteo*, *NFM_Basal*, *NFM+DEX_Osteo* and *NFM+DEX_Basal*, after 7, 14 and 21 days. Data were analyzed by nonparametric way of a Kruskal–Wallis test followed by Tukey's HSD test: a, denotes significant differences compared to *NFM_Osteo*; b, denotes significant differences compared to *NFM_Basal*; c, denotes significant differences compared to *NFM+DEX_Osteo*.

(*NFM+DEX_Osteo*), the expression of alkaline phosphatase (ALP) is similar to the one observed in control PCL NFM cultured on standard osteogenic differentiation medium (*NFM_Osteo*) ($p > 0.01$) (Fig. 7). An exception was observed for the 14 days of culture: hBMSCs cultured on DEX-loaded NFMs under dexamethasone-absent conditions (*NFM+DEX_Osteo*) produced significant higher quantities of ALP than the control PCL NFMs under osteogenic conditions (*NFM_Osteo*) ($p < 0.01$). Undifferentiated hBMSCs cultured on the control PCL NFMs (*NFM_Basal*) produce negligible quantities of ALP, as depicted by the significantly lower ALP concentration compared to all the other substrates and corresponding culture medium ($p < 0.01$). Consequently, further characterization of the osteogenic phenotype and genotype herein reported does not include this condition.

The morphology and distribution of the hBMSCs cultured on DEX-loaded NFM was analyzed by SEM. The cells interacted with the fibrous structure, bridging contiguous fibers and formed a dense cellular construct that was clearly visible at day 7 (Fig. 8A). It was also possible to observe deposition of minerals over the dense cellular layer from day 14 onward (Fig. 8B and C), resulting from the matrix mineralization that occurs along the differentiation of hBMSCs into osteoblasts. To confirm the matrix mineralization, an alizarin red staining was performed to specifically detect the calcium present in those mineralized nodules. The intense red/purple dots dispersed in the construct correspond to the mineralized nodules, after 14 and 21 days of hBMSCs culture on DEX-loaded NFM under dexamethasone-absent osteogenic differentiation medium formulation (Fig. 8D and E). Using Energy Dispersive

Spectrometry (EDS) it was possible to identify the elemental composition of the minerals deposited on the DEX-loaded PCL NFM-hBMSCs constructs (Fig. 8F). EDS data confirms the presence of Calcium and Phosphorous deposition by the adjacent cells, and the increasing amount from day 14–21 of hBMSCs culture under dexamethasone-absent osteogenic differentiation medium formulation. Those observations were not found in control PCL NFM cultured with hBMSCs under standard osteogenic differentiation conditions, nor in the DEX-loaded PCL NFM culture with hBMSCs on basal medium (data not included here).

The osteogenic phenotype of hBMSCs seeded onto DEX-loaded nanofiber meshes was also assessed by the immunodetection of some specific osteoblastic proteins, namely osteopontin, bone sialoprotein and osteocalcin. Photomicrographs indicate a progressive expression of the specific osteoblastic glycoprotein osteopontin on the DEX-loaded NFMs-hBMSCs constructs during the 21 days of culture (Fig. 9). In the cases of bone sialoprotein and osteocalcin protein, the maximum expression seems to be achieved at the 14th day of hBMSCs culture under dexamethasone-absent osteogenic differentiation medium. Similar osteoblastic protein expression pattern was observed in the control PCL NFM cultured with hBMSCs under standard osteogenic differentiation conditions and, also, in the DEX-loaded PCL NFM culture with hBMSCs in basal medium (data not shown).

Complementary to the phenotypic analysis, a genotypic quantification of the expression of some bone-specific genes was performed to ascertain about the differentiation level of the seeded hBMSCs on DEX-loaded PCL NFMs. The relative expression of those

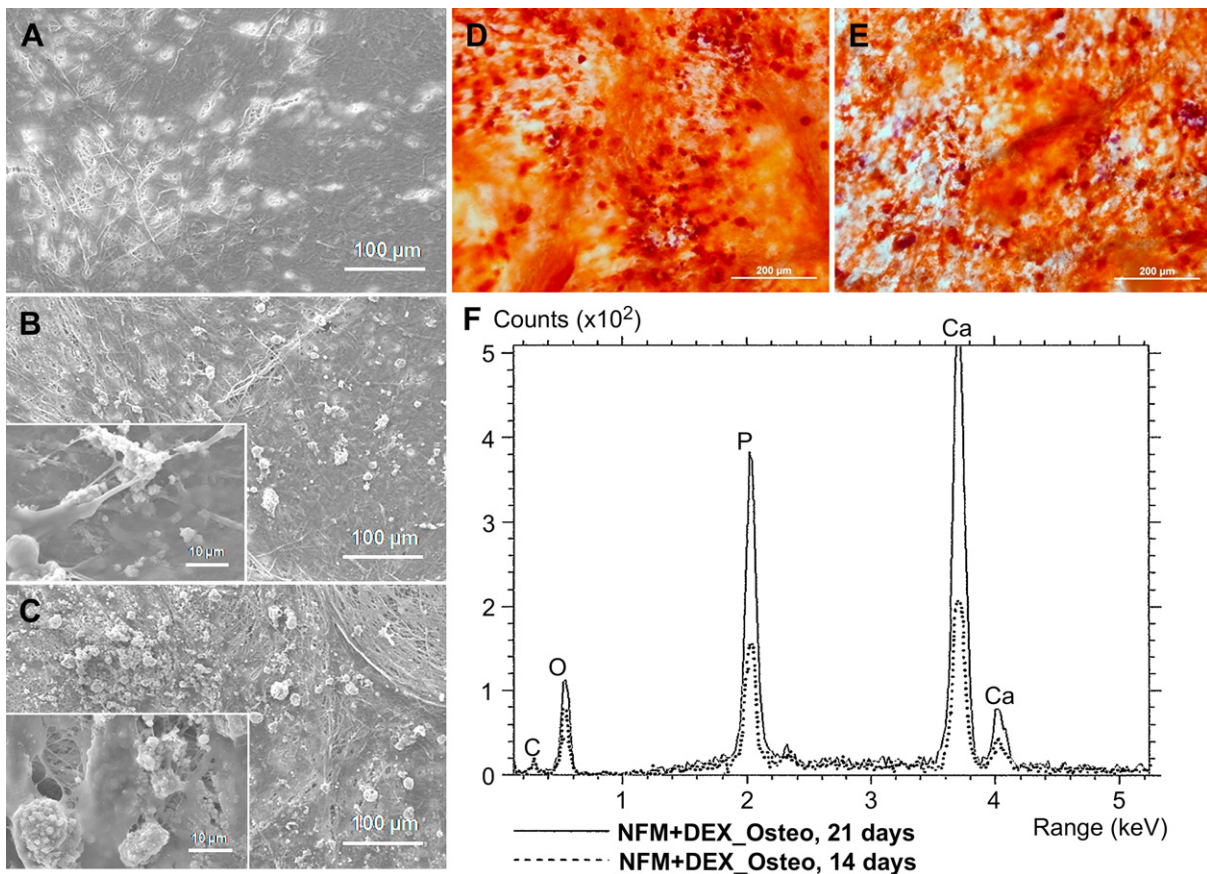


Fig. 8. SEM micrographs of hBMSCs cultured on DEX-loaded NFMs during 7 (A), 14 (B) and 21 days (C). Higher magnifications are shown to evidence the mineralization nodules produced by the hBMSCs. Alizarin red staining of the DEX-loaded NFMs-hBMSCs constructs after 14 (D) and 21 days (E). Energy dispersive spectrometer (EDS) spectra of the calcium phosphates nodules produced by hBMSCs cultured on DEX-loaded NFMs after 14 and 21 days (F).

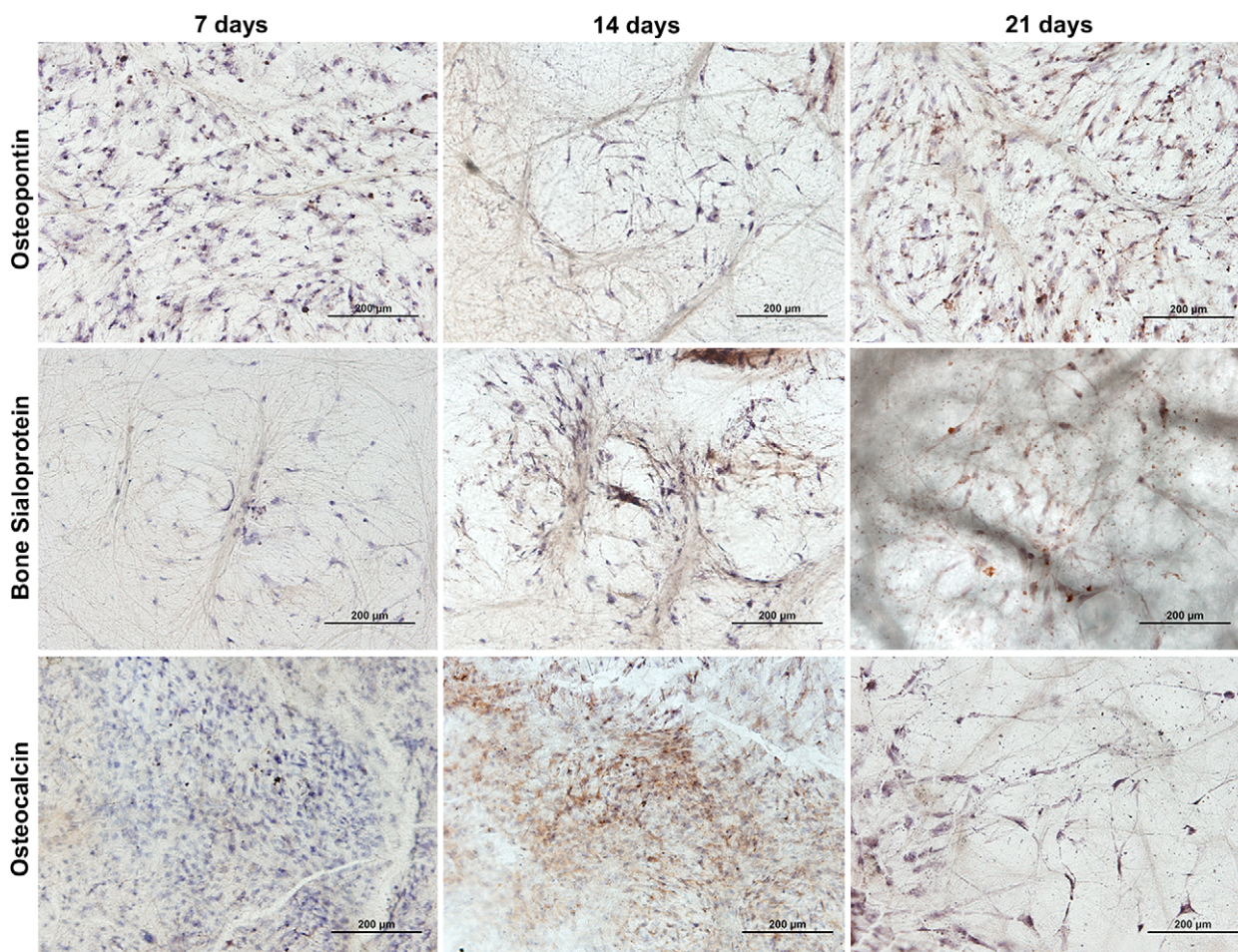


Fig. 9. Immunodetection of some bone-specific proteins, namely Osteopontin, Bone Sialoprotein and Osteocalcin, expressed by hBMSCs cultured on DEX-loaded NFMs during 7, 14 and 21 days.

genes was normalized against the housekeeping gene *GAPDH* and compared to hBMSCs cultured on control PCL NFM under standard osteogenic differentiation conditions. A constitutive expression of all mRNA transcripts (i.e. *Alkaline Phosphatase*, *Osteopontin*, *Bone Sialoprotein*, *Osteocalcin*, *Runx-2* and *Osterix*) was observed during the 21 days of culture. Although *Alkaline Phosphatase* was present in a stable fashion, its expression is significantly lower in hBMSCs cultured on DEX-loaded PCL NFMs, in both culture conditions ($p < 0.01$) (Fig. 10). Among those bone-specific genes, *Bone Sialoprotein* and *Osterix* were the highest expressed, followed by the *Osteopontin* gene.

4. Discussion

4.1. Electrospun nanofiber meshes incorporating and releasing dexamethasone

The rate of drug release depends on a multitude of factors, such as the nature of the polymer matrix (i.e. chemical composition, backbone stability and water solubility), matrix architecture, loading capacity and drug–matrix interaction. Therefore, for the development of an ideal drug release system, the polymer should be biodegradable, have appropriate degradation rate, nontoxic degradation products, appropriate solubility and simplicity of fabrication [30]. The herein developed drug carrier was based on the convenient processing of nanofibrous structures by

electrospinning, using an adequate organic solvent to simultaneously dissolve the biodegradable polymer – polycaprolactone – and the incorporated drug – dexamethasone. This drug release system allows a suitable interaction between PCL and dexamethasone, being the drug well distributed in the polymer matrix. Consequently, the drug presents a good physicochemical interaction with the polymer. The structural integrity of the differentiation factor, as well as its biological activity could be maintained during the processing step. The large surface area associated with the nanofiber mesh allows fast and efficient solvent evaporation, which limits the period of time of the incorporated drug to recrystallize, favouring the maintenance of amorphous dispersed phase [2,6,13]. The presence of amorphous dexamethasone in our polymeric carrier was also observed in our DSC data.

The most widely investigated release system for the encapsulation of bioactive compounds is micro- and nanoparticles. Nanoparticles have facility to diffuse through cell membranes, allowing controlling or modifying the cell activity. The osteogenic differentiation factor herein incorporated in electrospun biodegradable nanofibers was already studied by our group and others in different drug release systems, including micro- and nanoparticles and scaffolds [31–39]. Those polymeric drug release systems are based in starch–polycaprolactone (SPCL) microparticles or carboxymethylchitosan/poly(amidoamine) dendrimer (CMChT/PAMAM) nanoparticles. Both devices were intended to be used as part of an injectable system or in a polymeric 3D scaffold. In the present work

it was presented a dexamethasone release system, based on electrospun biodegradable nanofibers as carrier. It is aimed at developing a system that simultaneously acts as a scaffold for mesenchymal stem cells differentiation toward the osteogenic lineage. The next generation of engineered tissues relies on the development of biodegradable scaffolds as physical supports to release bioactive molecules that control cell attachment, proliferation and differentiation [40].

Due to the relatively slow wetting, as a consequence of the highly hydrophobic character of the PCL NFM and its slow degradation rate, it was possible to deduce that dexamethasone release occurred primarily by diffusion, demonstrating that a biodegradable nanofibrous system can be used to obtain a release profile that was effective in differentiating stem cells. The burst release effect common in polymer-based release systems, is connected to the high gradients of concentration observed at the initial stages of release. In the system of 20 wt.% DEX-loaded PCL NFMs, the burst release effect may be primarily caused by an imperfect distribution/entrapment of the dexamethasone into the polymer matrix or by their tendency to migrate to the nanofiber surface during the electrospinning process [41]. As the nanofibers are placed in an aqueous environment, at the initial stage water penetrates into the nanofibers surface and a large amount of dexamethasone is faster released due to the initial surface erosion. Subsequently, water permeates into the bulk of the nanofibers causing polymer swelling, bond cleavage and bulk erosion, generating interconnecting pores and channels within nanofibers

that allow the slow sustained release of the remaining dexamethasone [41].

4.2. Bioactivity of the dexamethasone loaded in the electrospun nanofiber meshes

Drug release systems are developed to maximize the therapeutic activity while minimizing the toxic side effects of drugs. However, the scope of those devices is limited for targeting tissues rather than individual cells [1]. In tissue engineering, the required bioactive molecules are typically supplied to the cells growing on the scaffold in the culture medium, in the soluble form. The present drug carrier was designed to operate as a synthetic extracellular matrix – scaffold – for mesenchymal stem cell growth and, simultaneously, to allow a direct supply of the differentiation factor to the adherent cells. It is intended to supply locally the required amount needed to promote the proliferation and differentiation towards the osteogenic lineage. To validate this hypothesis, an osteogenic differentiation study was conducted. The osteogenic differentiation is sensitive to the dose and duration of exposure to the glucocorticoid [42]. Jaiswal *et al.* suggested that the effective concentration of DEX for the osteogenic differentiation of MSCs should be in the range of 10–100 nM, showing toxic effects at 1000 nM [24].

The electrospun PCL NFMs loaded with 15 wt.% dexamethasone were cultured with hBMSCs under dexamethasone-absent

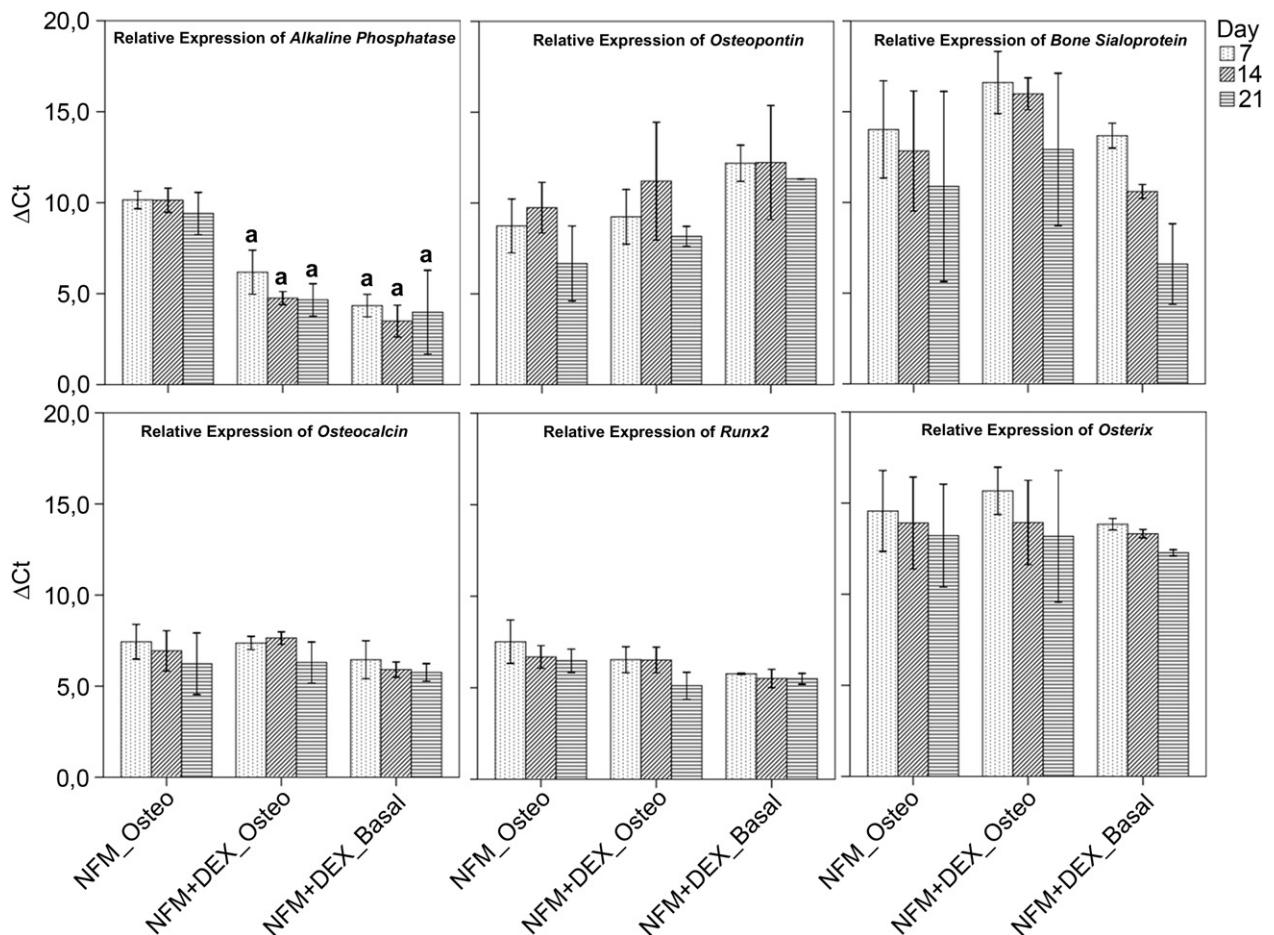


Fig. 10. Relative expression of bone-specific transcripts, namely *Alkaline Phosphatase*, *Osteopontin*, *Bone Sialoprotein*, *Osteocalcin*, *Runx2* and *Osterix*, by hBMSCs cultured on *NFM_Osteo*, *NFM+DEX_Osteo* and *NFM+DEX_Basal*, during 7, 14 and 21 days. The expression of these genes was normalized against the housekeeping gene *GAPDH* and calculated by the $\Delta\Delta C_t$ method. Data were analyzed by nonparametric way of a Kruskal–Wallis test followed by Tukey's HSD test: a, denotes significant differences compared to *NFM_Osteo*.

osteogenic differentiation medium formulation. Our biological results confirm the biological activity of the incorporated dexamethasone, as shown by the increased amount of deposited calcium phosphates produced by the differentiated cells (also stained by alizarin red and identified by EDS). Additionally, the increasing concentration of the enzyme alkaline phosphatase (ALP) also confirms the osteoblastic differentiation and mineralization of hBMSCs cultured on DEX-loaded PCL NFMs. In fact, this enzyme catalyses the splicing of phosphate from nonphosphoric esters, constituting a biochemical indicator of the presence of osteoblasts and the deposition of mineralized ECM, since MSCs produce negligible amounts of this enzyme, as depicted by our results. This hypothesis of osteoblastic differentiation was confirmed by the expression of specific osteoblastic glycoproteins, namely osteopontin and bone sialoprotein, which are also present in the ECM of bone. The deposition and mineralization of ECM, previously observed in the SEM micrographs, stained with alizarin red and chemically identified by EDS, were also confirmed by the immunodetection of the osteocalcin protein, which binds strongly to apatite and calcium.

In the literature three periods were identified on the osteogenic differentiation of embryonic stem cells (ES) [43], corresponding to the expression pattern of the osteoblastic markers: a proliferative phase, which is followed by the period of matrix deposition, and lastly the mineralization phase. The highest expression peak of *Osteopontin* mRNA at 14 days of hBMSCs culture determines the end of the matrix deposition phase and the beginning of the mineralization phase. The very high expression level of *Bone Sialoprotein* confirms the presence of mature osteoblasts, corresponding to the mineralization phase. Finally, this phase was confirmed by the expression of *Osteocalcin* mRNA, which is designed as the essential marker of the mineralization phase, reaching a maximum expression just before or during tissue mineralization [30]. Therefore, considering our results and the differences in the cell type used, the most important genes involved in the osteogenic differentiation process were constitutively expressed, confirming the matrix deposition and mineralization by hBMSCs cultured and differentiated on DEX-loaded NFMs. The main specific transcription factors involved in the osteogenesis were also quantified by qPCR, namely the *core binding factor α 1/runt-related gene (Cbfa1/Runx-2)* and *Osterix (Osx)*. *Cbfa1/Runx2* was already shown to be involved in the two steps of the differentiation process: driving stem cells into preosteoblasts and also preosteoblasts into osteoblasts. *Osterix* acts only during the last stage involving the transition preosteoblast/osteoblast [44,45]. Our data shows that *Osterix* and *Bone Sialoprotein* genes were the bone-specific transcripts with the highest expression levels. Those results further corroborate the successful differentiation of hBMSCs into osteoblasts on DEX-loaded NFMs. Similar osteoblastic gene expression patterns were observed for hBMSCs culture on DEX-loaded NFMs under basal conditions. This surprising result corroborates the hypothesis that even a transient exposure of stem cells to DEX (specifically during the 1st week) may be effective in inducing and maintaining the osteoblastic phenotype [24].

5. Conclusions

We report herein the development of electrospun biodegradable nanofibers as a release system of an established osteogenic differentiation agent of hBMSCs – dexamethasone. Dexamethasone was incorporated at different concentrations (5, 10, 15 and 20 wt.% polymer) in the polymeric nanofibers, being the 15 wt.% release system selected for cell studies because of its sustained release. An increased alkaline phosphatase concentration and deposition of mineralized matrix were observed on

dexamethasone releasing nanofibrous system cultured with hBMSCs in dexamethasone-absent osteogenic differentiation medium. The phenotypic and genotypic expression of osteoblastic-specific markers confirmed the osteogenic inducing potential of the loaded growth/differentiation factor. Thus, an osteogenic inductive scaffold was developed aimed for bone tissue engineering strategies.

Acknowledgments

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Appendix

Figures with essential color discrimination. Figs. 4, 8 and 9 in this article are difficult to interpret in black and white. The full color images can be found in the online version, at [doi:10.1016/j.biomaterials.2010.04.010](https://doi.org/10.1016/j.biomaterials.2010.04.010).

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