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ARTICLE

Immobilization of Bioactive Factor-Loaded Liposomes at the Surface of Electrospun Nanofibers Targeting Tissue Engineering

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Abstract

Electrospun nanofiber meshes (NFM), due to their morphology and fibrous structure, are extensively proposed as biomedical devices, tissue engineer on scaffolds and also as drug delivery systems. Liposomes are nanoparticles made by a biologically-derived material (phospholipid), already in clinical use as a drug release device. Liposomes may be combined with biomaterial scaffolds to promote a local and sustained delivery of loaded bioactive agents. The main objective of the present study is to evaluate the efficacy of dexamethasone (Dex)-loaded liposomes immobilized at the surface of electrospun polycaprolactone (PCL) NFM for promoting the osteogenic differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs). The *in vitro* release profile demonstrates a sustained release of Dex during 21 days, after an initial burst release of 12 h. Biological assays shows that Dex-loaded liposomes immobilized at the surface of electrospun PCL NFMs do not exhibit any cytotoxic effect, being able to successfully promoting the osteogenic differentiation of hBMSCs. We herein validate the concept

of using liposomes immobilized at the surface of a nanostructured fibrous system to be used as an advanced cell carrier device with autonomous release of growth/differentiation factors relevant for tissue engineering and regenerative medicine strategies.

1. Introduction

There is a huge need for more effective treatment modalities capable of regenerating tissues of damaged or diseased tissues. This important clinical need stimulates the development of new technologies in many fields, but particularly by the interdisciplinary areas encompassing Tissue Engineering and Regenerative Medicine (TERM). Therefore, by applying combinations of biomaterial scaffolds, cells and bioactive molecules the tissue regeneration can be guided and stimulated.[1] Biomaterial scaffolds were already previously developed to deliver cell regulatory signals such as growth differentiation factors and cytokines. Those scaffolds are powerful microenvironments to positively influence and guide the stem cell fate, both in ex vivo culture and also post-implantation *in vivo*.[2] Biomaterial scaffolds for TERM can be also used as reservoirs or release systems for bioactive molecules.[3-5] They can be functionalized to serve as local release systems modulating the activities of communities of cells in direct contact with the device including their coordinated differentiation.[6-8] Specifically, the drug delivery kinetics is particularly relevant when the therapeutic agent is a growth/differentiation factor aimed to achieve an effective dose and spatiotemporal release kinetics at the intended site of injury.[9, 10] Strategies that combine scaffolds and drug delivery systems have the potential to provide more effective tissue regeneration when compared with currently available therapies.[10]

Electrospinning has attracted a huge interest in the biomaterials research community as a simple and versatile technique enabling to produce polymeric ultrafine fibers with diameters ranging from a few micrometers down to tens of nanometers.[11, 12] Electrospun fiber meshes exhibit high specific surface area, excellent mechanical properties and flexibility in surface functionalities.[11, 13] Moreover, electrospun nanofiber meshes (NFM) enable mimicking the morphology of the extra cellular matrix of native tissues.[14] Electrospun NFM surfaces have been chemically functionalized to achieve sustained delivery of various bioactive molecules, just by physical adsorption.[12, 15] Optimized surfaces may be achieved in the nanofibers by gas plasma treatments[16], UV-Ozone treatments[17], wet chemical methods[15], surface graft polymerization[18], and co-electrospinning of bioactive agents and polymers.[19] Several bioactive molecules including antibiotics[20], siRNA[21], growth or differentiation factors[4, 22], anti-cancer drugs, enzymes and cytokines, have been entrapped within electrospun NFMs for controlled drug delivery.[15, 20] Electrospun NFMs have been also chemically modified to allow immobilization of specific cell ligands to enhance the

cell adhesion, proliferation and differentiation by mimicking the morphology and biological functions of the native natural ECM. [15, 23-25]

The most important advantages of combining TE scaffolds with growth factor-loaded nanoparticles are the possibility and flexibility of obtaining a well-controlled, sustained and local release. Examples of release systems are liposomes, dendrimers and polymeric nanoparticles.[1, 26-28] Immobilized nanoparticles do not only interact with target cells, but also with the substrates on which the cells are cultured. This combined release systems may be used to improve the therapeutic efficacy and safety of growth factors, by delivering them at the site of action and at a rate dictated by the need of the physiological environment.[29, 30] Indeed, it is well described that nanoparticles immobilization is thought to increase growth factor efficiency by increasing the local concentration closer to the cell surfaces and also increasing its bioavailability and half-life.[31, 32]

Liposomes have specific advantages in controlling the delivery of growth factors, when immobilized/incorporated in biomaterial scaffolds. Both the structure and the surface properties can be optimized for that specific application. An example is the incorporation of polyethylene glycol (PEG) molecules bearing terminal amine and thiol groups in the liposome surfaces. These modified liposomes were already covalently bound onto stainless steel disks.[33] Other studies described the sustained release systems that use liposomes loaded with proteins or drugs and further incorporated into fibrin sealants.[34-36] Using these carriers it is possible to maintain therapeutic drug levels at the defect site, minimizing the total amount of drug required when compared with systemic administration dose and its side effects.[3, 37] Three different model drugs (carboxyfluorescein, doxorubicin, and lysozyme) were previously encapsulated into liposomes and immobilized into collagen/hydroxyapatite composite scaffolds.[37] The drugs entrapped into the liposomes showed a slow release from scaffolds. Mickova et al. demonstrated that electrospinning does not allow processing intact liposomes.[38] Alternatively, coaxial electrospinning allowed the successful incorporation of liposomes into the nanofibers.[38]

Cell culture devices using this new approach may be efficiently used to control the behavior of stem cells. MSCs hold a great potential in several therapies due to their unique biological characteristics, such as self-renewal and capability to differentiate into specific cells. These attributes make them excellent candidates for cell and tissue regeneration therapies.[4, 39-41] Herein, we developed multi-functionalized electrospun polycaprolactone (PCL) NFMs with the potential to be used as stem cells differentiation inductive structures, through the covalent immobilization of drug-loaded liposomes carrying Dex. For that, electrospun PCL NFMs were functionalized using UV-Ozone irradiation and aminolysis, followed by the insertion of thiol groups using 2-iminothiolane (2IT). PCL is hydrophobic and lacks the functional groups intended for liposome immobilization. The UV-Ozone irradiation increases the hydrophilicity of the polymer surface by generating polar groups such as -OH and –COOH.[42] The polar groups can be advantageous for the insertion of NH2 groups by aminolysis and further insertion of the thiol groups,

when used for liposome immobilization. Furthermore, we intended to analyze the effect of Dex-loaded liposomes immobilized at the surface of electrospun PCL NFMs on the viability, proliferation, protein synthesis and differentiation of human bone marrow-derived mesenchymal stem cells (hBMSCs).

2. Results

2.1. Development and characterization

One aim of this study was to develop a method to generate functional groups at the surface of electrospun PCL NFMs, which provide binding sites suitable to immobilize drug-loaded liposomes. Previously, we optimized the encapsulation of Dex into the liposomes (formulation F1).[49] In this work, we used the optimized liposome formulation, and we added DSPE-PEG-Mal to the liposome formulations (F2 and F3) to bind to the generated functional groups. In the **Table 1**, it is presented the various liposome formulations used in this study (see the description in the experimental section). The formulation F1 was used to perform the biological assays from liposomes in suspension. The formulations F2 (with cholesterol - Chol) and F3 (without Chol) were used for the analysis of the presence of liposomes immobilized at the surface of the PCL NFM by florescence microscopy and also for the biological assays. **Figure 1** shows a scheme of the liposome formulations and the chemical pathway followed for the covalent immobilization of the Dex-loaded liposomes at the surface of PCL NFM (*NFM4_F2* and *NFM4_F3*).

Table 1- Liposome formulations (values expressed as a molar ratio)

	Dex	**HSPC**	**Chol**	**DSPE-PEG**	**DSPE-PEG-Mal**	**PE-Rho**
F1	0.25	2	0.1	0.1		0.02
F2	0.25	2	0.1	0.04	0.06	0.02
F3	0.25	2	-	0.04	0.06	0.02

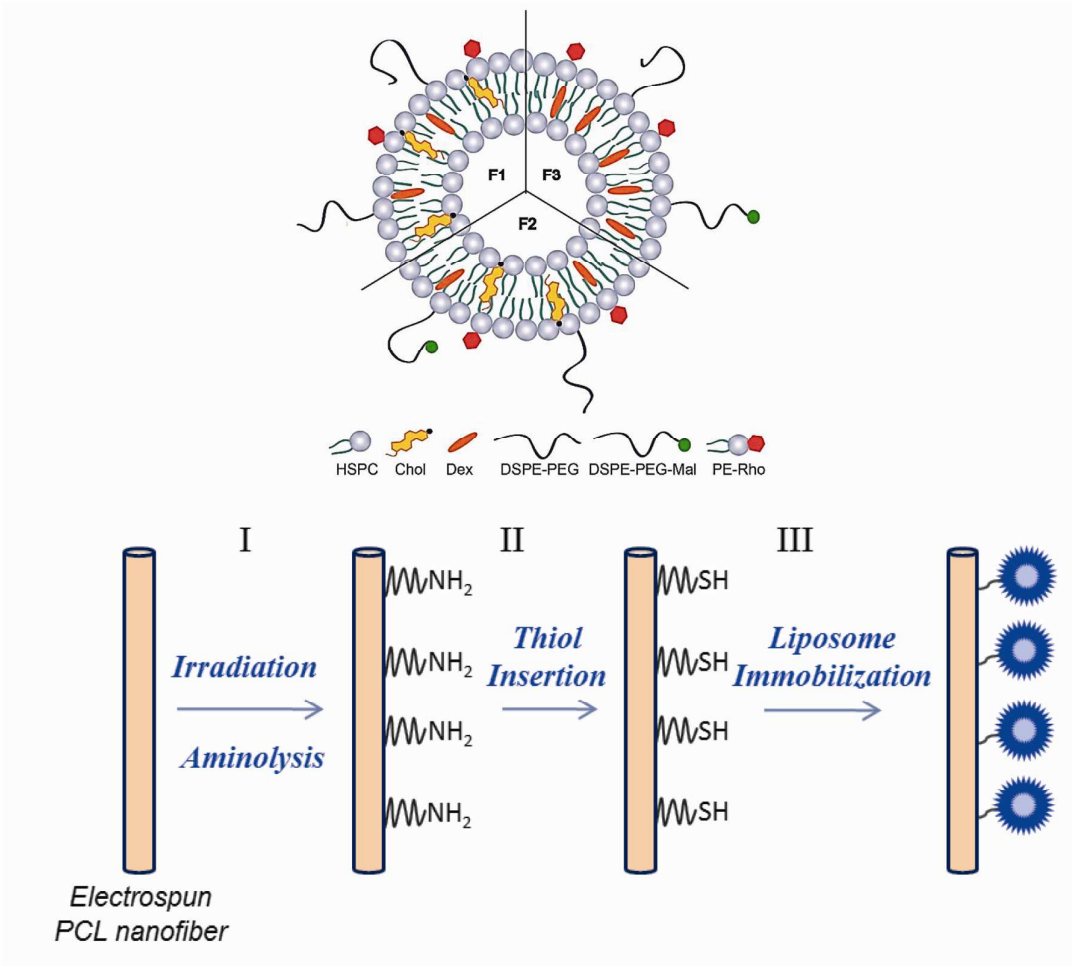

Figure 1 – Liposome formulations and chemical pathway used for the immobilization of liposomes at the surface of modified PCL NFM (*NFM_F2* and *NFM_F3*). I – UV-Ozone irradiation and aminolysis treatment, II – primary amine group insertion and 2-iminothiolane reaction (at pH 8), III – thiol group insertion and maleimide reaction (at pH 7).

2.1.1. SH Groups Quantification

The binding sites suitable to immobilize drug-loaded liposomes were achieved by creating SH groups at the surface of electrospun PCL NFM. This was achieved by creating SH groups at the surface of electrospun PCL NFM. In order to evaluate the impact of the different processing steps, the amount of free SH groups introduced at the surface of the NFMs was determined by the Ellman's reagent method.[43] **Table 2** presents the quantity of SH groups at the surface of the modified NFMs as a function of the UV-Ozone irradiation time. The concentration of free SH groups at the surface of electrospun NFMs increased with the irradiation time from 1 to 5 min, as compared to the control sample (t=0 min, no SH was detected). The SH maximum concentration was achieved at 5 min of UV-Ozone irradiation.

Table 2 – Quantification of the free SH groups at the surface of activated NFM, as a function of UV-Ozone irradiation time.

UV-Ozone time (min)	SH concentration nmol cm^{-2}
0	0
1	0.60 ± 0.09
2	1.17 ± 0.09
3	1.87 ± 0.37
4	2.83 ± 0.11
5	2.94 ± 0.11

The UV-Ozone irradiation time was selected taking into account the morphology and the concentration of SH groups. If the selection would be only based in the quantification of SH groups at the surface our choice would be 5 min UV-Ozone treatment time. However, this treatment has an impact on the morphology of the meshes, thus the selection of the UV performance was set at 4 min. Other parameters may affect the efficiency of grafting the SH groups at the surface of the NFMs, namely the reaction time of the aminated surface with 2IT (used to generate the free SH groups) and of the SH-functionalized surface with DTNB (used to quantify the SH groups). The effect of both parameters is shown in the **Table 3**. The effect of the DTNB seems to be higher than the effect of 2IT. Given those results the condition selected was 3 h 2IT and 3 h DTNB.

Table 3 – Effect of 2IT and DTNB reaction time in the SH concentration at the surface of electrospun NFM

2IT (h)	DTNB (h)	SH / NH_2 nmol cm^{-2}
1	**1**	1.62 ± 0.17
2	**2**	3.15 ± 0.40
3	**1**	2.11 ± 0.17
1	**3**	5.01 ± 0.42
3	**3**	5.52 ± 0.53

2.1.2. Morphology of activated and functionalized NFMs

The **Figure 2** shows the micrographs of the PCL NFMs treated by UV-Ozone and aminolysis. It is observed from the SEM micrographs that the integrity of the fibers at the surface is significantly affected by the UV-Ozone irradiation. The integrity of the fibers decreases for longer treatment time (4 and 5 min). The aminolysis treatment further affects the integrity of the fibers at the surface, being its effects visible for shorter UV-Ozone irradiation time (2 min). 4 min UV-Ozone irradiation and 1 h aminolysis was the optimum condition selected since the concentration of the SH group was near to the maximum SH concentration (**Table 2**) and the integrity of the fibers was less affected.

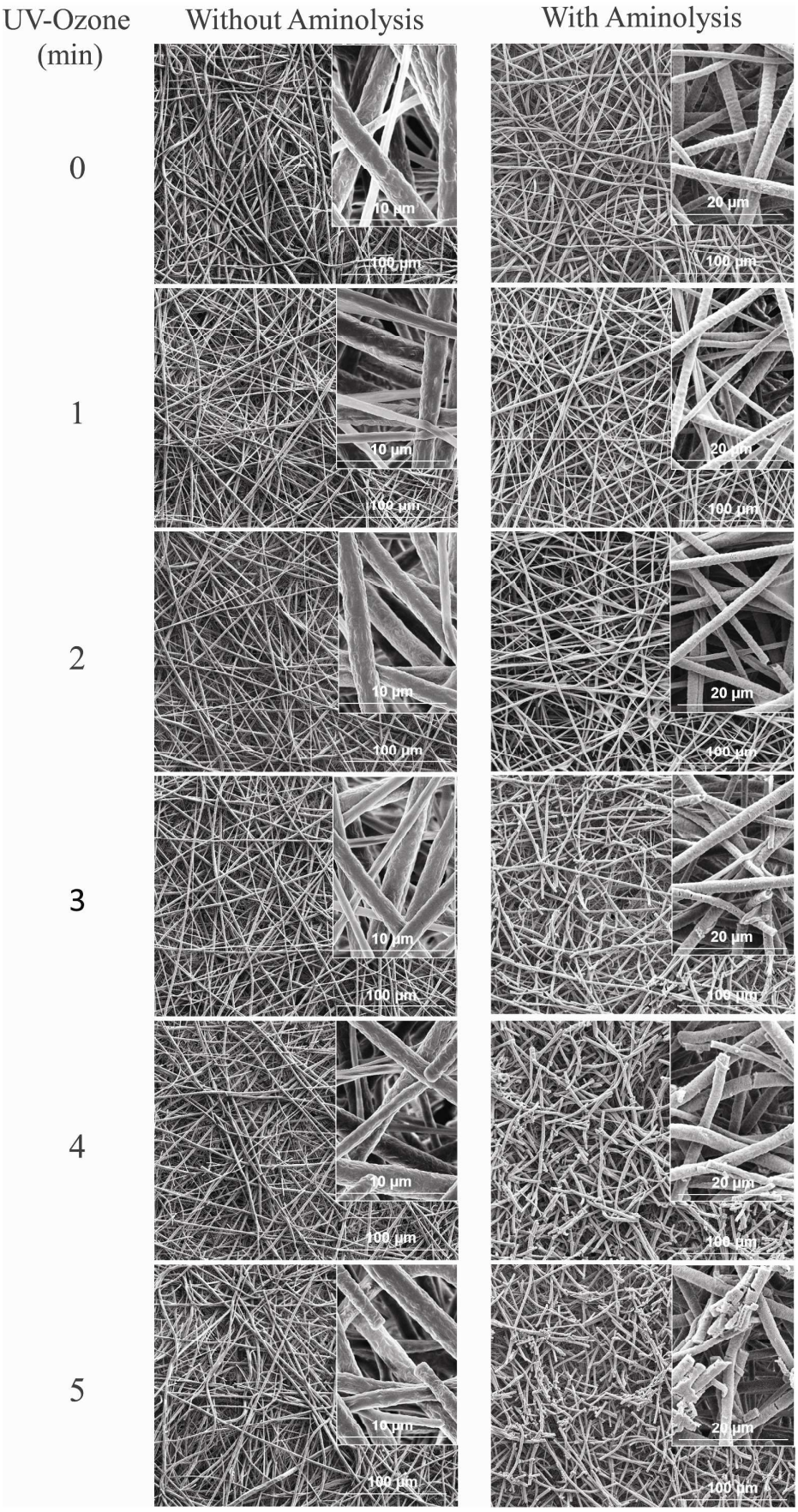

Figure 2 - SEM micrographs of UV-Ozone irradiated NFMs (exposure time ranging from 0 to 5 min) without and with aminolysis (t=1 h).

2.1.3. Distribution of SH groups at the surface of electrospun NFMs

The spatial distribution of the SH groups at the surface of electrospun PCL NFMs was evaluated by fluorescence microscopy using a SH-reactive probe, namely the fluorescent dye BODIPY. In the negative control, without BODIPY (**Figure 3**A), no auto fluorescence was detected, and only a slight fluorescent background (non-specific labeling) was observed on the negative control with BODIPY (**Figure 3B**). The observed uniform distribution of the binding sites at the NFMs' surface (**Figure 3C**) demonstrates that the SH functionalization step was successful and uniformly functionalized the whole surface of the NFM.

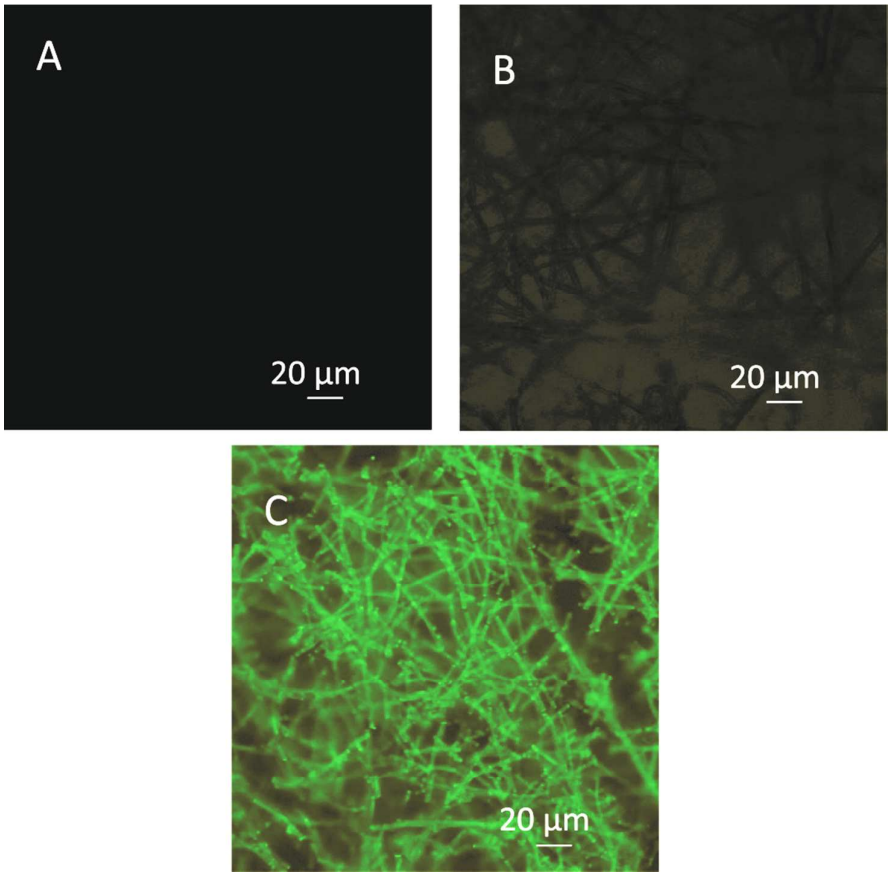

Figure 3 - Fluorescence micrograph of the electrospun PCL NFM (A); negative control (NFM) exposed to BODIPY but without SH groups (B) and SH-functionalized NFM reacted with BODIPY (C).

2.1.4. Liposome size and ζ-potential

Table 4 presents the size and ζ-potential of each of the synthesized liposomal formulations. The ζ-potential of liposomes is negative due to the presence of terminal carboxylic groups in the lipids DSPE-PEG. The size of the Dex-loaded liposomes showed a monodisperse distribution with diameters varying between 103.70 and 119.10 nm.

Table 4 – ζ-potential and size of Dex-loaded liposomes

Formulation	ζ-potential	Size (nm)
F1	-23.10±1.68	103.70±15.74
F2	-22.50±3.40	119.10±6.57
F3	-19.96±2.60	113.20±6.11

2.1.5. SEM and EDS analysis of Dex-loaded liposomes immobilized at the surface of electrospun NFMs

Figure 4 presents the SEM, EDS and fluorescence micrographs of Dex-loaded liposomes immobilized at the surface of electrospun PCL NFMs. For specific immobilization (**Figure 4B)**, the PCL NFMs were firstly chemically activated and functionalized as described in the experimental section. In the SEM micrograph (**Figure 4B**), it can be observed the presence of one liposome (diameter of 110 nm) immobilized at the surface of the NFM. The specific immobilization of the DSPE-PEG-Mal at the surface of electrospun NFM was confirmed by EDS analysis as shown in the spectra (**Figure 4C**), by the presence of the elements Carbon, Oxygen, Sodium, Phosphorus and Chloride. To ascertain about the uniformity of the immobilization of liposomes we analyzed by fluorescence the distribution of PE-Rho liposomes (i.e. liposome formulation F3) linked to the SH groups present at the surface of electrospun PCL NFMs (**Figure 4D**). The fluorescence image shows that the PE-Rho liposomes are evenly distributed at the surface of the activated and functionalized NFMs. Conversely, in the negative control in which the liposomes were presented to unfunctionalized NFM (inset of **Figure 4D**), no fluorescence signal could be detected, indicating that no auto fluorescence is detected in the NFMs.

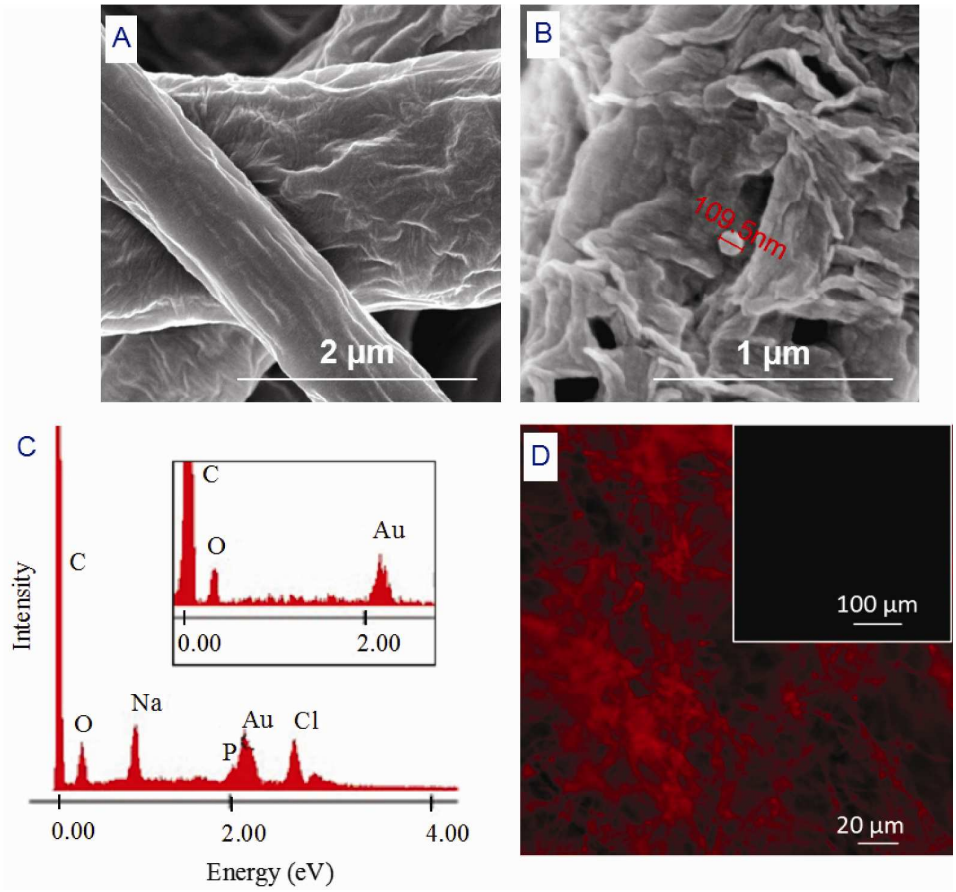

Figure 4 – SEM, EDS and fluorescence micrographs: A – NFM without liposomes; B – detail of a liposome (F3) immobilized at the surface of functionalized NFMs (t=4 min); C – EDS of the surface of electrospun NFM showing the presence of phosphors (P) (absent in the inset represent the EDS of NFM without liposome immobilized); D - Fluorescence micrograph of PE-Rho marked liposomes immobilized at the surface of SH functionalized NFM (inset represents negative controls without PE-Rho liposomes).

2.1.6. Affinity of the Dex-loaded liposomes to the surface of electrospun NFMs

We studied the affinity of the Dex-loaded liposomes to the SH-functionalized NFMs. For that, after the immobilization of Dex-loaded liposomes, the concentration of Dex was determined. The effect of the presence of cholesterol in the liposome formulation, the liposome size, the volume of liposomes in solution and the washing time on the maximum capacity of Dex-loaded liposome immobilized at the surface of the NFMs were all studied (Table 5). The results show that the excess of Dex-loaded liposomes not having access to SH groups is removed at the first washing step. The absence of Chol in the liposome formulation increased the concentration of Dex released from liposomes from 2.91±0.75 to 11.00±0.92µM. The liposome size had a direct effect on the Dex-loaded liposome concentration. Specifically, when the liposome size was increased from 100 to 400 nm, the Dex concentration increased from 11.00±0.92 to 18.76±0.92 µM. Increasing 3 times the volume of the liposome solution, the Dex concentration increased to 31.46±4.12 µM without any washing, and further decreased to 19.65±1.54 µM after the first washing step.

Table 5 – Concentration of Dex immobilized at the surface of NFMs, considering the effect of the presence/absence of cholesterol, membrane pore size, volume of liposomes and washing times on the capacity Dex liposome immobilization.

Sample	Cholesterol (ratio)	Nominal Size	Volume of Liposomes	Washing	Dex (μM)
NFM_F2	**0.1**	**100 nm**	**1.5 mL**	**1**	2.91±0.75
NFM_F3	**0**	**100 nm**	**1.5 mL**	**No**	15.98±2.25
				1	11.00±0.92
				2	11.05±0.34
				3	11.38±0.65
		400 nm	**1.5 mL**	**No**	20.95±0.73
				1	18.76±0.92
				2	17.77±1.32
				3	17.70±0.65
		100 nm	**4.5 mL**	**No**	31.46±4.12
				1	19.65±1.54

Those results show that the SH groups were saturated, and some of the liposomes were not covalently linked to the nanofiber mesh surface. Thus, the amount of liposomes immobilized is specifically controlled by the amount of SH groups available at the surface, being the remaining liposomes removed by the first washing. The conditions selected for further Dex release and biological tests were: liposomes immobilized at the surface of the activated and functionalized NFMs with cholesterol (*NFM4_F2*) and without cholesterol, with average size of 100 nm, 4.5 mL and 1 washing (*NFM4_F3*).

2.1.7. Dex release from liposomes immobilized at the NFMs' surface

The Dex release profile from the liposomes in suspension (formulation F1) and immobilized at the surface of activated and functionalized NFMs (*NFM4_F2* and *NFM4_F3*) was followed during 21 days (Figure 5). This time frame was selected in accordance with the culture time required to obtain the osteogenic differentiation of MSCs *in vitro*. The release profile of the Dex is characterized by an initial burst release, lasting 12 hours. Afterwards, a slower but sustained release rate was observed during the remaining time. The release rate of liposomes immobilized at the surface of NFM (*NFM4_F3*) is in cumulative terms more effective than the one observed for the other conditions, being probably more effective in leading to the osteogenic differentiation. This kinetics of release is adequate to validate the concept proposed in our study.

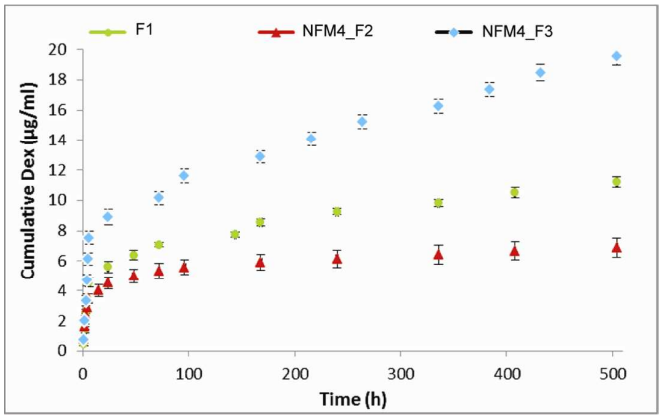

Figure 5 - *In vitro* cumulative Dex release from liposomes in suspension (*F1*) and immobilized at the surface of activated and functionalized NFMs with and without cholesterol (*NFM4_F2* and *NFM4_F3*).

2.2.Biological Activity

The effectiveness of this multifunctionalized systems were assessed by culturing hBMSCs under osteogenic differentiation medium. Table 6 describes the experimental conditions used in the biological assays (see the description in the experimental section).

Table 6 - Experimental conditions used in the biological assays

Condition	Description
NFM0_Ost	Non-functionalized PCL NFMs in standard osteogenic medium
NFM0_F1	Non-functionalized PCL NFMs with Dex-loaded liposomes (F1) in suspension in Dex-absent osteogenic differentiation medium
NFM4_Ost	SH-functionalized PCL NFMs in standard osteogenic medium
NFM4_F2	SH-functionalized PCL NFMs with Dex-loaded liposomes (F2 with Chol) immobilized at the surface in Dex-absent osteogenic differentiation medium
NFM4_F3	SH-functionalized PCL NFMs with Dex-loaded liposomes (F3 without Chol) immobilized at the surface in Dex-absent osteogenic differentiation medium
NFM_5%Dex	Non-functionalized PCL NFMs loaded with 5% of Dex in Dex-absent osteogenic differentiation medium

2.2.1. Cell viability and proliferation assessment

The influence of Dex-loaded liposomes immobilized at the surface of electrospun nanofibers over hBMSCs viability and proliferation was assessed using standard cell biology protocols, namely the MTS and the PicoGreen assays, respectively. In terms of cell viability (**Figure 6**), during 14 days, the hBMSCs culture on the 4 min UV-Ozone irradiated NFMs under osteogenic differentiation medium (NFM4_Ost) displayed significantly higher viability (Abs 490 nm) than all other culture conditions (Kruskal-Wallis test, Tukey's HSD test, $p<0.001$). However, at the 21st day, the NFM4_Osteo presents significantly

higher hBMSCs' viability than untreated NFMs cultured under osteogenic differentiation medium supplemented with Dex-loaded liposomes with cholesterol (NFM0_F1) ($p<0.001$). Likewise, NFM4_Osteo presents significantly higher hBMSCs' viability than Dex-loaded liposomes with Cholesterol immobilized at the surface of UV-Ozone treated NFMs cultured under osteogenic differentiation medium (NFM4_F2) and 5% Dex encapsulated into electrospun nanofibers (NFM_5%Dex) ($p<0.001$). It is also notable that the presence of liposomes in solution (NFM0_F1) or immobilized at the surfaces, either with cholesterol or not have cell viability at the level of the untreated NFM during all the culturing period. The viability after 7 days of culture is lowest when Dex is loaded and released from the nanofiber meshes (NFM_5%Dex). It is surprising the result obtained for the condition (NFM4_Ost), having the highest viability even compared with untreated meshes in osteogenic supplemented medium (NFM0_Ost). The viability of the hBMSCs when cultured at the surface of the NFM functionalized and having liposomes is comparable to that observed in non-functionalized NFMs. This is a very important result.

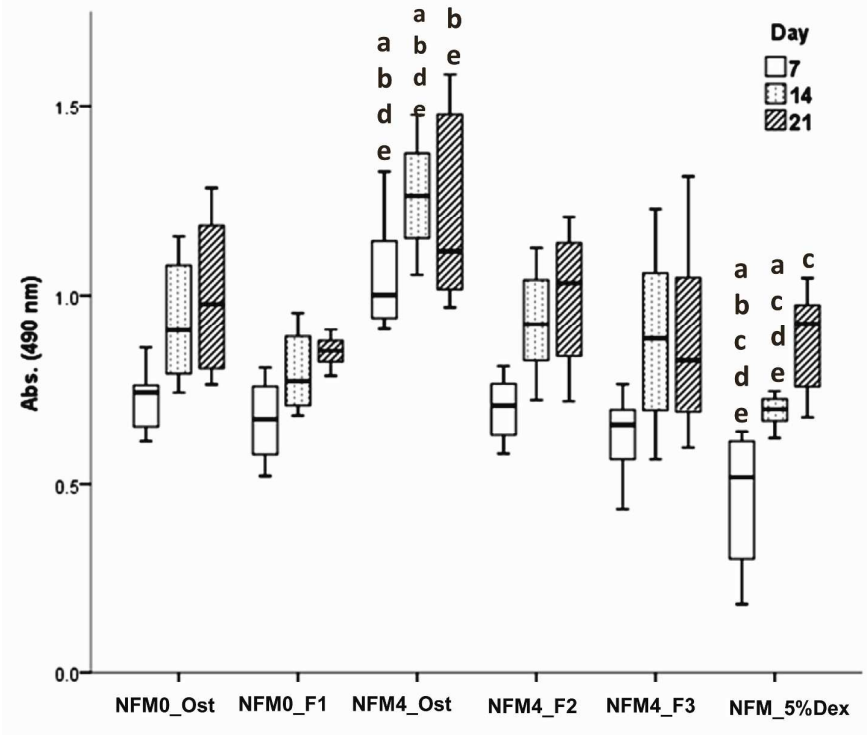

Figure 6 - Box plot of hBMSCs' viability (i.e. Abs. (490 nm)) when cultured on NFM0_Ost, NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex during 7, 14 and 21 days. a denotes significant differences compared to NFM0_Ost, b denotes significant differences compared to NFM0_F1, c denotes significant differences compared to NFM4_Ost, d denotes significant differences compared to NFM4_F2 and e denotes significant differences compared to NFM4_F3.

In terms of cell proliferation, (**Figure 7**), at 7th day the hBMSCs' culture conditions Dex-loaded liposomes without cholesterol immobilized at the surface of UV-Ozone treated NFMs (NFM4_F3) and the NFM_5%Dex displayed significantly lower DNA concentration values than all other culture

conditions ($p<0.001$). The same culture conditions (i.e. the NFM4_F3 and the NFM_5%Dex) also displayed significantly lower DNA concentration values for the 14th and 21st days of culture than the NFM4_Ost and the NFM4_F2 ($p<0.001$). Inversely, hBMSCs cultured on the NFM4_Ost displayed significantly higher proliferation than NFM0_Ost, NFM4_F3 and NFM_5%Dex ($p<0.001$) for the 14th and 21st days. This result suggests that an effective release of Dex at the surface of the NFMs may interfere with the metabolic activity of the cells in those conditions. The supplementation of the medium or the release of Dex from liposomes in suspension seems not to have a similar effect over the proliferation of cells.

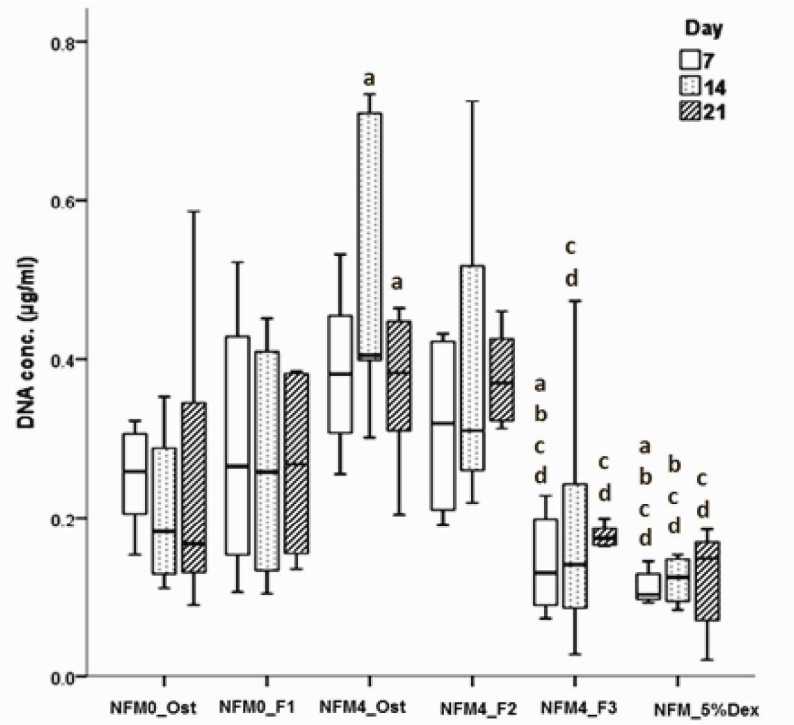

Figure 7 - Box plot of the hBMSCs' proliferation (i.e. DNA conc. (µg/ml)) when cultured on NFM0_Ost, NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex during 7, 14 and 21 days. *a* denotes significant differences compared to *NFM0_Ost*, *b* denotes significant differences compared to *NFM0_F1*, *c* denotes significant differences compared to *NFM4_Ost*, *d* denotes significant differences compared to *NFM4_F2*.

2.2.2. Alkaline Phosphatase Activity quantification

In order to assess the onset of the osteoblastic activity of the cultured hBMSCs, the quantification of the alkaline phosphatase enzyme activity was performed according to a p-nitrophenol assay (**Figure 8**). For the 7th day of hBMSCs culture, the condition NFM0_F1 displayed a significantly higher ALP activity than the conditions NFM0_Ost, NFM4_Ost and NFM4_F2 ($p<0.001$). Additionally, the culture condition NFM4_F3 displayed a significantly higher ALP activity than the conditions NFM0_Ost and NFM4_Ost ($p<0.001$). Those results show that our concept of releasing the Dex at the surface where the cells are attached is more effective in promoting the osteogenic differentiation. For the 14th day, the

culture condition NFM0_F1 displayed a significantly higher ALP activity than all other culture conditions ($p<0.001$). The culture condition NFM4_F2 displayed a significantly lower ALP activity than the other conditions indicating that the Dex release rate may be insufficient to drive the osteogenic differentiation in this condition. Similarly, at the 21st day of hBMSCs culture, the condition NFM0_F1 displayed a significantly higher ALP activity than the conditions NFM0_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex ($p<0.001$). The culture condition NFM4_F2 displayed a significantly lower ALP activity than the conditions NFM0_Ost, NFM0_F1, NFM4_Ost and NFM4_F3 ($p<0.001$). It can be shown from the ALP activity data that the liposomes are more effective in stimulating the osteogenic activity of hBMSCs, with the exception of the condition using Chol that limits the encapsulation of Dex.

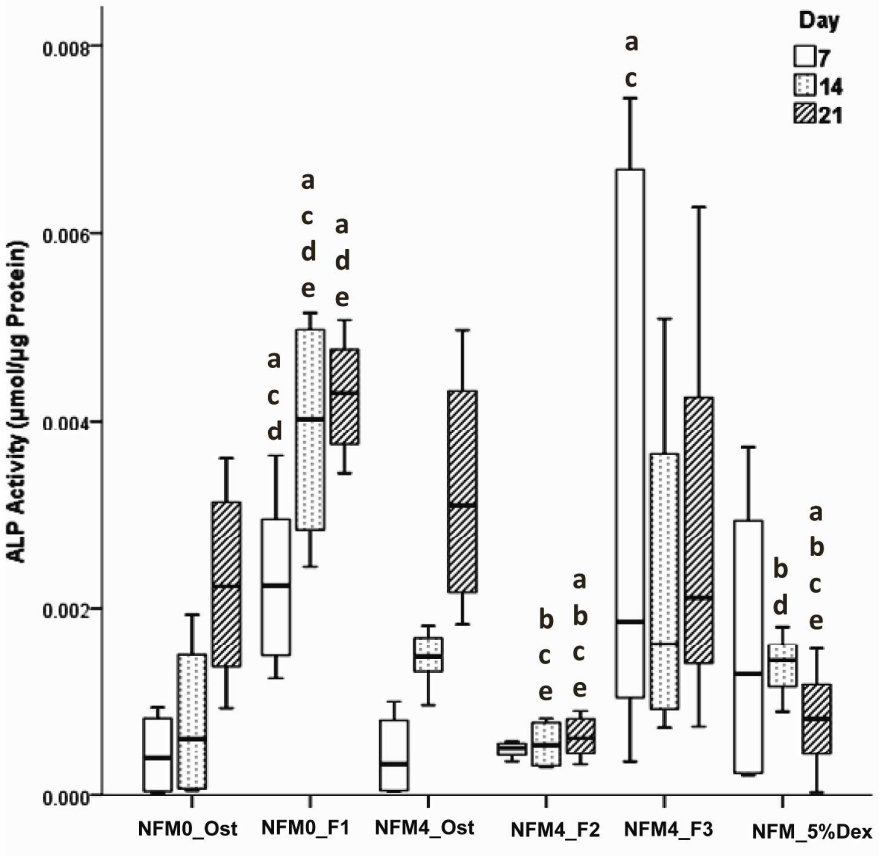

Figure 8 - Box plot of the ALP activity (µmol/h/µg Protein) from the hBMSCs cultured on *NFM0_Ost,* NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and *NFM_5%Dex* during 7, 14 and 21 days. *a* denotes significant differences compared to *NFM0_Ost*, *b* denotes significant differences compared to *NFM0_F1*, *c* denotes significant differences compared to *NFM4_Ost*, *d* denotes significant differences compared to *NFM4_F2* and *e* denotes significant differences compared to *NFM4_F3*.

2.2.3. Genotypic expression of Osteoblastic markers

In terms of ALP, OP, OCN, Runx2, Osterix expressed (**Table 7, Figure 9**), no statistically significant differences were found between the various conditions at the 7th, the 14th and the 21st days of culture. Also, no significant differences were found between the culturing periods for the conditions NFM0_Ost, NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex. However, when

comparing the gene expression patterns of NFM4_F3 with the condition supplemented with osteogenic medium (NFM0_Ost and NFM4_Ost) the condition releasing Dex from liposomes immobilized at the surface is at least as effective in promoting the expression of osteogenic genes as the supplemented cultures. This result suggests that our strategy is at least as effective as the traditional osteogenic inducing methods.

Table 7 – P values between the culturing days for the conditions NFM0_Ost, NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex, and between the material for the 7th, the 14th and the 21st day.

Conditions	ALP	OP	OCN	Runx2	Osterix
NFM0_Ost	0.023	0.283	0.190	0.584	0.296
NFM0_F1	0.018	0.888	0.126	0.117	0.231
NFM4_Ost	0.105	0.034	0.500	0.551	0.905
NFM4_F2	0.049	0.036	0.041	0.176	0.184
NFM4_F3	0.013	0.397	0.044	0.034	0.020
NFM_5%Dex	0.852	0.757	0.400	0.543	0.311

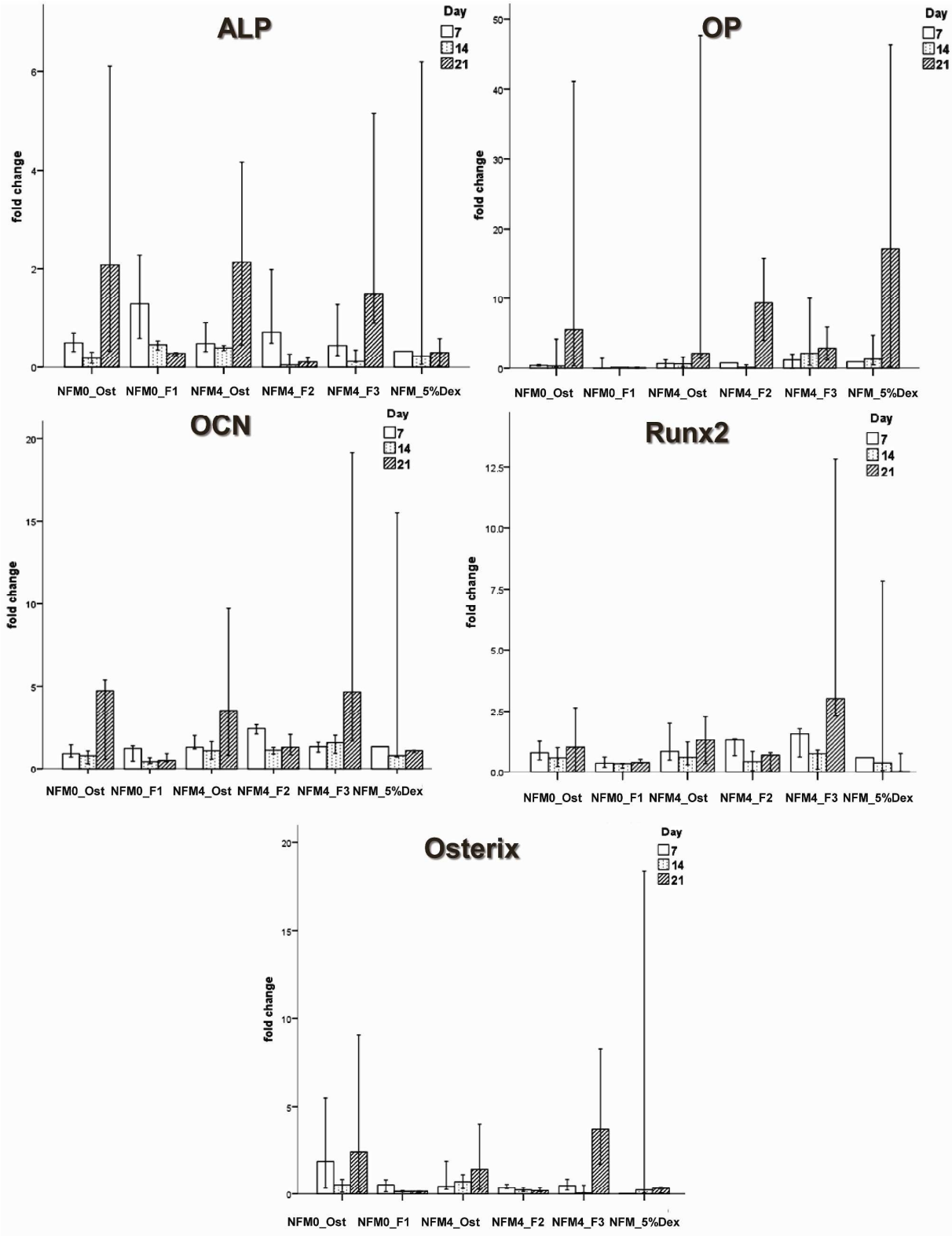

Figure 9 - Bar plot of the ALP, OP, OCN, Runx2, and Osterix in NFM0_Ost, NFM0_F1, NFM4_Ost, NFM4_F2, NFM4_F3 and NFM_5%Dex.

3. Discussion

Indeed, both we[4] and others already reported the loading of dexamethasone (as a model drug) in electrospun meshes, showing its efficacy in differentiating stem cells. The present manuscript describes a very different concept, in which we immobilize at the surface of the PCL electrospun substrate liposomes releasing at the surface, locally, dexamethasone. This innovative method is needed since it enables functionalizing the surface of polymeric substrates (any polymeric substrate where we can find or insert amine groups) enabling releasing a bioactive agent at the surface where the cells are attached. As such, this work shows much enhanced versatility in the range of substrate materials where it can be applied and the liposomes were already shown to be able to carry many different types of drugs (including hydrophilic and hydrophobic), supporting the claim that this strategy may be applied for many different strategies. The advantages of our system, besides its versatility, include a substantial reduction of the total amount of drug used, maximizing its efficacy. The drug is not affected by the electrospinning process, not requiring exposure of the drugs to the solvents used in its processing.

3.1 Development and characterization

Herein, we report the chemical modification of electrospun PCL NFMs enabling the immobilization of Dex-loaded liposomes onto their surfaces. To achieve this purpose, initial UV-Ozone irradiation was used to generate reactive free radicals that were immediately subjected to aminolysis. These modified surfaces were reacted with 2IT to generate sulfide (SH) pendant groups. Dex-loaded liposomes were covalently bonded to the SH groups present at the surface of electrospun NFMs. The availability of the drug-release vehicle at the surface of the NFMs (where initial cellular contact occurs) enables a sustainable release of the loaded drug at the vicinity of the cells in culture and, consequently, increasing its efficacy and bioavailability. The activation and functionalization study demonstrated that the concentration of free SH groups at the surface of NFMs increases with the UV-Ozone irradiation time (**Table 2**). The optimum SH concentration is achieved at 4 min of UV-Ozone irradiation. Moreover, the effect of 2IT and DTNB reaction times in the grafting of SH groups was determined. Analyzing the effect of both factors on the quantification of SH groups (**Table 2**), the impact of the variation in *t* (DTNB) is more relevant than *t* (2IT). Therefore, and in accordance to the data presented in the SEM micrographs (**Figure 2**), the UV-Ozone irradiation and aminolysis have a significant effect on the integrity of the electrospun PCL NFM causing shortening of the fibers. The damage to the nanofibers is mainly due to the aminolysis reaction. In fact, this effect has been also detected and previously reported in the literature[44] in amorphous biodegradable aliphatic polyester nanofibers. However, the morphology of the nanofibers present in the bulk of the mesh underneath the surface is not significantly affected neither by the irradiation nor by the chemical treatment. A BODIPY fluorescent dye was covalently bound to the SH groups present at the surface of electrospun NFMs, and their spatial distribution was characterized by

fluorescence microscopy as uniform throughout the NFMs surfaces. **Figure 3C** shows the homogeneous distribution of SH functionalization at the surface of the irradiated and chemically functionalized electrospun NFMs.

The ζ-potential analysis revealed that the liposomes are negatively charged due to the presence of the terminal carboxylic groups in the lipids DSPE-PEG (**Table 4**). Indeed, the negative ζ-potential of PEGylated liposomes is positive since it avoids agglomeration and stabilizes the liposome suspension.[45] The size of the Dex-loaded liposomes showed a monodisperse distribution, with diameters ranging between 103.70 (with PE-Rho) and 119.10 nm (without PE-Rho). The DSPE-PEG-Mal lipids comprising the outer layer of the liposomes facilitates the binding to the SH groups grafted at the surface of electrospun NFMs. Specifically, the maleimide group (Mal) reacts with SH groups at pH ranging from 6.5 to 7.5, forming a non-reversible and stable thioether link. The particular efficiency of surface-bonded PEG chains is explained by the steric repulsive barrier around the liposomes provided by the covalently bonded PEG.[46] Some methods were previously proposed to determine the incorporated amounts of PEG-derivatized lipids in liposomes for the physicochemical characterization of PEG-coated liposomes.[47] PEGylated liposome formulations were developed for cancer treatment, showing to be efficient in stabilizing drug-loaded liposomes, allowing to bind to proteins and to increase their stability in the blood circulation.[48] The EDS analysis (**Figure 4E**) confirms the presence of the element phosphorous that is derived from the characteristic phosphate group of the phospholipids used to produce the liposomes. The spatial distribution of the liposomes at the surface of electrospun NFMs was evaluated by fluorescence microscopy emitted by the rhodamine dye (**Figure 4F**). This analysis showed that the immobilization at the surface of Dex-loaded liposomes was successful and evenly distributed throughout the surface of the NFM.

The effect of the presence of cholesterol (Chol) in the liposome formulation, washing time, liposome size and volume in solution to get the maximum capacity of Dex-loaded liposome immobilized at the surface of electrospun PCL NFMs were all studied (**Table 5**). Our data demonstrated that the presence of Chol has a major negative effect on the encapsulation efficiency of Dex. Specifically, the concentration of Dex increased when Chol was removed from the liposomes formulation, which is in accordance with the results from previous reports in the literature (e.g. [49]). Moreover, one washing step was sufficient to remove the liposomes not covalently linked to the reactive SH groups. Liposomes produced with 400 nm were shown to enable increasing the loaded Dex. Previous reported data shows that the extrusion process of the liposomes reduces the efficiency of drug encapsulation.[50]

The release behavior of Dex from the liposomes immobilized at the surface of NFMs showed an initial burst release, which ended after 12 h (**Figure 5**). Afterwards, a slower but steady Dex release was observed during the remaining period of study. Jaiswal *et al.* [51] suggested that the effective concentration of Dex for the osteogenic differentiation of MSCs should be kept within the range of 100 to 1000 nM,

showing toxic effects above 1000 nM. Our results are consistent with the availability of Dex at the surface of electrospun NFMs (where the initial cell attachment occurs), in a concentration relevant for inducing the osteogenic differentiation of MSCs. To confirm this hypothesis, further biological assays with human bone marrow-derived mesenchymal stem cells (hBMSCs) were conducted.

3.2 Biological Activity

The effect of Dex-loaded liposomes immobilized at the surface of PCL NFMs on the viability, proliferation, protein synthesis and osteogenic differentiation of hBMSCs were all assessed by the use of standard protocols. Biological data shows that immobilized Dex-loaded liposomes (NFM4_F2 and NFM4_F3) are not cytotoxic to hBMSCs (**Figure 6**). Thus, the performance of the PCL NFMs when the liposomes are immobilized at the surface does not affect the performance of the meshes. Moreover, they are able to induce an earlier osteogenic differentiation of hBMSCs since the 7th day of culture as depicted by the results of ALP activity (**Figure 8**) and by gene expression patterns (**Figure 9**) for NFM4_F3. This significant result is in accordance with our previous report where Dex-loaded liposomes were used in solution to induce the osteogenic differentiation of hBMSCs on tissue culture plates.[52] This observation is of statistically significant relevance when compared to the control culture condition NFM_Ost or to the incorporation of Dex into electrospun NFM (NFM_5%Dex), for the in vitro induction of hBMSCs osteogenic differentiation. Those previously explored strategies need 14 to 21 days to get the hBMSCs fully differentiated into osteogenic lineage. In a previous work, Martins et al. [4] tested the bioactivity of the released Dex by cultivating hBMSCs on 15% Dex-loaded PCL NFMs, under dexamethasone-absent osteogenic differentiation medium formulation. The early osteogenic differentiation of the hBMSCs at the 7th day of culture was not observed, as well as on the control condition NFM_5%Dex. Somehow this relevant effect may be related with the release of Dex by the liposomes at the vicinity of the cells in culture, contributing to the direct interaction between the liposomes and the cells, and even to the possible uptake of the liposomes by the cells into the intracellular space. Dex-loaded liposomes in suspension, i.e. NFM0_F1 showed a better biological performance than the control condition NFM0_Ost regarding the ALP activity. However, the early osteogenic differentiation of the hBMSCs at the 7th day of culture was also not observed in that condition.

The biological results show a positive physicochemical effect of the activation by irradiation and further chemical functionalization of electrospun NFMs under osteogenic differentiation conditions (NFM4_Ost) when compared with untreated NFMs. The surface activation and functionalization improved the biological performance of PCL NFMs in the osteogenic differentiation of hBMSCs when compared to the control supplemented osteogenic medium culture condition (NFM0_Ost). It is well described in the literature that some functional groups, such as amine and thiols, influence positively the adhesion, proliferation and differentiation of stem cells. [53, 54] Our data further confirms that hypothesis.

The concentration of Dex in the culture medium and the mode of release are important factors in the successful differentiation of hBMSCs.[9, 51] By using the liposomes immobilized at the surface of electrospun PCL NFMs, we hypothesized a benefit by releasing the loaded bioactive agent at the vicinity of the cells, increasing its bioavailability without the need of further supplementation of the culture medium. Above, we described that the concentration of Dex decreases when Cholesterol is used in the liposomes formulation. This explains the low ALP activity observed when hBMSCs were cultured on NFM4_F2. Also, this result can explain the low ALP activity observed when hBMSCs were cultured on NFM_5%Dex, which is at the lower level of Dex concentration required to differentiate hBMSCs.[51] By giving repeatedly Dex-loaded liposomes in suspension, NFM0_F1, the ALP activity increased as described above. However, for implantation in vivo into a bone defect as a release system, it would not be practical to use this modality. By using NFM4_F3, we could implant our functionalized NFM into a bone defect as an autonomous device, and the released Dex would be sufficient to promote the in situ osteogenic differentiation of attracted MSCs minimizing the risk of undesirable negative side effects caused by a systematic administration dose.

Liposomes have been used with some success in tissue engineering, such as in an animal model for cartilage repair, avoiding the limited efficacy of the direct injection of growth factors into the synovial cavity.[55] Moreover, the conjugation of liposomes with a scaffold could improve the bioactive agent release efficiency.[55] Also, the nanofibers with embedded growth factors-loaded liposomes showed an enhancement of MCSs proliferation.[38] Therefore, this multi-functionalized and versatile carrier for drugs or different growth/ differentiation factors system may also be used to transfect cells *in vivo*.

4. Conclusions

The present work proposes the use of surface immobilized Dex-loaded liposomes in a strategy to confer electrospun PCL NFMs the autonomous capability to support the osteogenic differentiation of hBMSCs. The PCL NFM surface was activated and functionalized by UV-Ozone irradiation and aminolysis, respectively. Optimal functionalization was obtained with 4 min UV-Ozone irradiation time and 1h of subsequent aminolysis. Dex-loaded liposomes were covalently immobilized at the surface of electrospun NFMs, and the release kinetics showed a steady release of Dex (until 21 days) after an initial burst period of 12h. The developed system is intended to act as a drug carrier that can be designed to operate as a synthetic support for MSC growth and, simultaneously, to allow a direct supply of the differentiation factor (e.g. Dex) to the adherent cells. To validate this hypothesis, an osteogenic differentiation study was conducted using human bone marrow-derived mesenchymal stem cells (hBMSC). Biological data showed that the Dex-loaded liposomes immobilized at the surface of electrospun PCL NFMs exhibited no cytotoxicity and promoted the effective osteogenic differentiation of hBMSC when compared to the osteogenic supplementation of the culture medium. Concluding, we herein

validate the concept of using liposomes immobilized at the surface of a nanostructured system to be used as an autonomous medical device for the local and sustained release of growth/differentiation factors relevant for bone regenerative strategies.

5. Experimental Section

5.1 Production and characterization

5.1.1 Materials

Polycaprolactone (PCL), dexamethasone (Dex), 1,6-hexamethylenediamine (HMD), isopropyl alcohol (IPA), 4-(dimethylamino)pyridine (DMAP), N,N-dimethylformamide, chloroform, ammonium molybdate, fiske-subbarow reducer, Ellmans reagent (DTNB), 2-iminothiolane (2IT), sepharose CL4B, hepes buffer solution (HBS), Tween 20 used in this study were reagent grade purchased from Sigma-Aldrich. The lipids L-α-Phosphatidylethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium Salt) (Egg-Transphosphatidylated, Chicken) (PE-Rho), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG), cholesterol (ovine wool, >98%) (Chol) and L-α-phosphatidylcholine, hydrogenated (Soy) (HSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG-Mal) were purchased from Avanti Polar Lipids. Boron-dipyrromethene (BODIPY, 493/503 FL N-(2-aminoethyl)maleimide) fluorescent dye was purchased from Invitrogen, USA. All the materials were used as received.

5.1.2 Preparation of electrospun PCL NFM

The production of electrospun PCL NFMs was performed as described in detail elsewhere.[4] In brief, a polymeric solution of 17% w/v PCL in chloroform and N,N-dimethylformamide (7:3 volume ratio) was electrospun at 8-10 kV, using a needle-to-ground collector distance of 20 cm, and a flow rate of 1.0 mL/h. The NFMs were collected in a flat vegetal foil, and the solvents were evaporated under vacuum. Dex-loaded PCL NFMs were produced by adding 5 wt.% Dex/PCL to the polymeric solution and electrospun, as described elsewhere.[4] Those Dex-release nanofiber meshes were used as controls in the biologic assays.

5.1.3 Activation and functionalization of PCL electrospun NFM surface

Figure 1 shows a scheme of the chemical pathway followed for the immobilization of the Dex-loaded liposomes at the surface of PCL NFM. The PCL NFM surfaces were activated by UV-Ozone irradiation by using the ProCleaner system, which emits UV light at 254 nm (intensity 10–13 mW cm^{-2}) and 185 nm (10% intensity of the 254 nm line). The same treatment time was applied to both sides of the PCL NFMs, from 1 to 5 min. The reaction between the activated surfaces and HMD was performed in order to insert amine functional groups onto the NFM surfaces. To this purpose, the NFMs were immersed in 1M HMD solution and incubated for 1h at 37°C (step I). Afterwards, SH groups were inserted at the surface of the NFMs (step II) through the reaction of the amine groups with 20 mM 2IT solution (0.1 mM PBS at pH 8, and with 20 mM DMAP) solution for 1h at 37ºC. The efficiency of SH

insertion onto the NFM surfaces was quantified using the Ellman's reagent method.[43] The samples were immersed in 0.1 mM DTNB solution (in PBS 0.1 mM at pH 7.27) and incubated for 1 h at 37ºC. Thereafter, the UV absorbance of the supernatant (in triplicate) at 412 nm (Synergie HT) was used to quantify the SH groups, using the DTNB solution as blank. For the calculation the 2-nitro-5-thiobenzoate (NTB^{-2}) molar absorption coefficient, the value of 14151 $M^{-1}cm^{-1}$ was used. It was performed an optimization of the thiol insertion. The UV-Ozone irradiation was maintained constant at 4 min, while the 2IT and DTNB reaction time was varied. We evaluated the impact of these two parameters in the SH-functionalization reaction and in the determination of the SH concentration at the NFM's surface. The morphologies of the activated and chemically functionalized NFMs were characterized by scanning electronic microscopy (SEM; NanoSEM, Nova 200). Prior to this analysis, the samples were coated with gold/palladium using a Cressington Sputter Coater 208 HR device. To assess the distribution of SH groups at the surface of electrospun NFMs, three samples of SH-functionalized PCL NFMs were immersed in a pre-prepared fluorescent BODIPY (493/503 FL N-(2-aminoethyl)maleimide) solution (0.4 mM BODIPY in PBS at pH 7). The SH-functionalized PCL NFMs were incubated for 4 hours at room temperature. As negative control, untreated samples were immersed in an equivalent solution. Afterwards, both types of NFMs were washed three times for 5 minutes with Tween 20 washing solution (0.5% (v/v) Tween 20 and phosphate buffer at pH 7) by gentle shaking. As the BODIPY is sensitive to light, all the steps were carried out under light protection. Binding of fluorescent dye to the NFMs was further analyzed by fluorescence microscopy (AXIOIMAGE RZ1M, ZEISS, Germany).

5.1.4 Preparation and characterization of liposomes encapsulating Dex

In the **Table 1**, it is presented the various liposome formulations used in this study. The formulation F1 was used to perform the release studies from liposomes in suspension and for the biological assays. The formulations F2 and F3 were used for the analysis of the presence of liposomes immobilized at the surface of the PCL NFM by florescence microscopy and also for the biological assays. The production of Dex-loaded liposomes was performed as described previously elsewhere in detail.[49] Briefly, lipids and Dex were mixed in a round-bottomed flask, dispersing proper amounts of each lipid from the stock solutions (15 mM total lipid), in the proportions described for each type of liposome formulation. The solvent, chloroform, was slowly evaporated using a gentle stream of nitrogen. The obtained dry film was dispersed using vortex agitation with HBS, keeping the temperature of the hydrating medium above the gel-liquid crystal transition temperature (T_c = 52°C). The multilamellar liposomal suspension was extruded at $T > T_c$ using a porous polycarbonate membrane (pore size of 100 nm and 400 nm) held between two tight syringes. The syringes were used to force the solution back and forth (21 times), resulting in unilamellar liposome vesicles. Non-encapsulated Dex was removed from the solution by column chromatography (Sepharose CL4B, Sigma Aldrich, Portugal) using an isocratic elution with HBS. Particle size distribution and ζ-potential were determined by dynamic light scattering (Zetasizer

Nanoseries ZS, Malvern Instruments, Portugal). The morphology of Dex-loaded liposomes immobilized at the surface of electrospun PCL NFM was analyzed by SEM (NanoSEM Nova 200, London). By EDS (EDS, Pegasus X4M), it was performed an elemental analysis of the NFMs to further confirm the presence of the liposomes at the surface of the NFMs.

5.1.5 Binding and Affinity of the Dex-loaded liposomes to the surface of electrospun NFM

SH-functionalized PCL NFMs were immersed in 2 mL of a liposome HBS solution (pH 7) containing rhodamine fluorescent dye (i.e. liposome formulation F2) for 4 hours at room temperature. Afterwards, the NFMs were washed three times with 3% ethanol in phosphate buffer (pH 7.4). As the rhodamine is sensitive to light, all the steps were carried out under light protection. Binding of the liposomes, carrying the fluorescent dye to the SH-functionalized NFMs was analyzed by fluorescence microscopy (AXIOIMAGE RZ1M, ZEISS, Germany).

The amount of Dex and lipids present in the NFMs were quantified to evaluate their affinity to the NFM surfaces. After liposome immobilization, 12 PCL NFMs were immersed in a solution of ethanol for 24 h to dissolve the lipids. The solutions were analyzed by high performance liquid chromatography (HPLC, KNAUER, Germany) using: an Atlantis T3 5 μm C-18 column; a flow rate of 1 mL/min; 0.2% phosphoric acid: acetonitrile (55:45) as the mobile phase; and UV detection at 247 nm. Dex was quantified using a calibration curve obtained with standard solutions, ranging from $4x10^{-4}$ to 0.1 mM. The total lipid concentration was assessed by the Bartlett colorimetric assay, as described elsewhere. [56] The principle of the Bartlett assay is based on the colorimetric determination of inorganic phosphate. The phospholipid content of liposomes can be determined after the destruction of the phospholipid with perchloric acid to obtain the inorganic phosphate. The inorganic phosphate is converted to phospho-molybdic acid by the addition of ammonium molybdate, which is reduced to a blue colored complex by 4-amino-2-naphthyl-4-sulfonic acid during heating. This compound can be determined colorimetrically at 830 nm. For the maximum capacity of Dex-loaded liposome immobilized at the surface of the NFMs, the effect of the presence of cholesterol, washing time, the liposome size and volume of liposomes in the solution were all studied. The experiments were performed in triplicate, and each specimen was also analyzed by HPLC (KNAUER, Germany).

5.1.6 Dex release from liposomes immobilized at the surface of electrospun PCL NFM

The release of Dex from the loaded liposomes in suspension (formulation *F1*) was studied using a dialysis method. Dialysis cellulose tubes (100-500 MWCO, Spectrum Labs, USA) were rinsed with distilled water for one week prior to their use. Tube ends were closed with Teflon tape and Nylon thread and tested for leakage. 1 mL of each liposome solution (~17% EE) was added to the dialysis tubes and

fully immersed into 10 mL of PBS as release medium. Control tubes were assembled using the same procedure with Dex-free liposomes. The solutions were maintained at 37ºC and 60 rpm for 21 days. For each time point, an aliquot of 1 mL was collected from the solution, and replaced with fresh PBS. The Dex release kinetics from the Dex-loaded liposomes (formulation F1) and Dex-loaded liposomes immobilized at the surface of electrospun PCL NFM with and without cholesterol (NFM4+F2 and NFM4+F3) was studied by immersion of NFMs specimens in 3 mL PBS. Aliquots of 300 μL were extracted from the solution at each time point and replaced with fresh PBS. The Dex concentration at each time point was determined by HPLC (KNAUER, Germany) as described above.

5.2 Biological Activity

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

hBMSCs were isolated from bone marrow aspirates collected under informed consent from patients undergoing knee arthroplasty at the Hospital de Braga, Portugal, in accordance with a protocol established between the 3B's Research Group and the Hospital de Braga approved by the ethics committee of the same Hospital. The bone marrow sample was collected from a 58 year old female donor, isolated, expanded and cryopreserved until further use. hBMSCs were isolated and characterized according to the method established by Delorme and Charbord.[57] Briefly, the plastic adherent fraction of marrow cells characterized by a spindle-shape morphology and colony-forming unit (CFU) capacity; positive expression of the surface antigens CD 29, 73, 90 and 105, and negative for the hematopoietic markers CD 34 and 45 (all the antibodies were purchased from BD Pharmingen (USA) and the hBMSCs were analyzed on a FACS Calibur, BD Biosciences (USA)); and characterized for the differentiation ability into the osteogenic, chondrogenic and adipogenic lineages. hBMSCs were expanded in basal medium consisting of MEM alpha medium (α-MEM; Gibco, GB) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, GB) and 1% antibiotic/antimyotic solution (final concentration of penicillin 100 units/mL and streptomycin 100 mg/mL; Gibco, GB). Cells were cultured at 37˚C in a humidified atmosphere of 5% CO_2. Confluent hBMSCs at passage 4 were harvested for seeding onto Dex-loaded liposomes immobilized at the surface of 1x1 cm^2 electrospun PCL NFMs (*NFM4_F2* and *NFM4_F3*) at a density of $1x10^5$cells/mesh. After 24 h of incubation in basal medium, hBMSCs were cultured under Dex-absent osteogenic differentiation medium (basal medium supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerophosphate) in the presence of Dex-loaded liposomes (*NFM0_F1*). Those liposomal solutions were sterilized by 0.22 μm filtering. SH-functionalized and non-functionalized PCL NFMs were used as control (*NFM4_Ost, NFM0_Ost*). The control condition (*TCPS_osteo*) was cultured under standard osteogenic differentiation medium (basal

medium supplemented with 50 μg/mL ascorbic acid, 10 mM β-glycerophosphate and 10^{-7} M Dex). The constructs were retrieved at the predefined culturing times, after 7, 14 and 21 days. All experiments were performed in triplicate and were repeated twice independently.

5.2.2 Cell viability and proliferation assessment

Cell viability for each culturing condition and time point was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, USA), according to the instructions of the manufacturer. This assay is based on the bioreduction of a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfofenyl)-2H-tetrazolium [MTS], into a water-soluble brown formazan product. The absorbance was measured at 490 nm in a microplate reader (SynergieHT, Bio-Tek, USA), being the cell viability directly related with the quantity of formazan product. Four independent analyses were performed and quantified.

Cell proliferation was quantified by the total amount of double-stranded DNA, along the culturing time. Quantification was performed using the Quant-iT™ Pico- Green dsDNA Assay Kit (Invitrogen™, Molecular Probes™; Oregon, USA), according to the instructions of the manufacturer. Briefly, hBMSCs within the construct were lysed by osmotic and thermal shock, and the supernatant was 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). Quadruplicates 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 the amount of DNA to the fluorescence intensity.

All data concerning cell viability, proliferation and ALP activity was independently measured and normalized against the cell number for each sample. For that, a standard calibration curve was performed using known hBMSCs cell numbers at passage 4, ranging from 0 to $5x10^5$ cells, with an n=12. The dsDNA concentration of these samples was determined according to the Quant-iT™ PicoGreen® dsDNA Reagent, previously described. The following equation was obtained: $y = 0.0054x + 86.68$ with a $R^2 = 0.998$, where the y is the measured fluorescence value and the x is the cell number, and used to estimate the cell numbers for each measurement.

5.2.3 Alkaline Phosphatase and Total Protein quantification

The concentration of alkaline phosphatase (ALP) was determined for all culturing periods, using the same cell lysates used for DNA quantification. Briefly, the ALP quantity was assessed using the *p*-nitrophenol assay, in which 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma, USA) is hydrolysed by the intracellular ALP at the temperature of 37 ˚C in an alkaline buffer solution (1.5 M and pH 10.5, Sigma) to form yellow free *p*-nitrophenol. The reaction was stopped by addition of 0.3 M NaOH (Panreac Quimica, Spain) and the absorbance was read at 405 nm in a microplate reader (Bio-Tek,

Synergie HT, USA). Standards were prepared with 10 mM *p*-nitrophenol (pNP; Sigma, USA) solution, to obtain a standard curve ranging from 0 to 250 μM. Quadruplicates of each sample and standard were made, and the ALP concentrations were read off directly from the standard curve.

For the quantification of total protein synthesized by the hBMSCs in culture, the Micro BCA™ Protein Assay kit (Thermo Scientific, Pierce; Rockford, USA) was used according to the manufacturer instructions. This is a colorimetric detection and quantification method which utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1} formed when Cu^{+2} is reduced by protein in an alkaline environment. Quadruplicates of lysed cells per culturing time were incubated at 37ºC for 2 h. A purple-colored reaction product was measured at 562 nm in a microplate reader (SynergieHT, Bio-Tek, USA) and calculated based on an albumin standard curve, ranging from 0 to 40 μg mL^{-1}.

5.2.4 RNA isolation and Real-Time Quantitative Polymerase Chain Reaction

At each culturing time, the hBMSCs were washed with PBS, immersed in Tri® reagent (Sigma-Aldrich, USA) and stored at -80 ºC until further use. Proteins were removed with chloroform:isoamylalkohol (BioChemica, AppliChem; Germany) extraction and the RNA pellets were washed once with 2-propanol (Sigma-Aldrich; USA) and once with 70% ethanol (Panreac; Spain). The total RNA pellets were reconstituted in Rnase free water (Gibco, Invitrogen; UK). Determination of the RNA concentration for each replica (quadruplicates of each condition per time point) was performed by microspectrophotometry (NanoDrop 1000, Thermo Scientific; USA).

Reverse transcriptase (RT)-PCR was performed according to the protocol from iScript™ cDNA synthesis kit (Quanta BioSciences™; Gaithersburg, MD). Briefly, a reaction mixture consisting of 1X iScript Reaction Mix, 1 μL iScript Reverse Transcriptase, 100 ng RNA template 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 22 ºC, followed by 30 min at 42 ºC and terminated by an incubation at 85 ºC for 5 min.

Amplification of the target cDNA for real-time PCR quantification were performed according to manufacturer, using 2 μL RT cDNA products, 250 nM each primer (bone-specific primer sets listed [4]) 1X PerfeCta® SYBR® Green FasterMix® (Quanta BioSciences™; Gaithersburg, MD) and nuclease-free water, in a final volume of 25 μL. Forty-four cycles of denaturation (95 ºC, 10 s), annealing (temperature dependent on the gene, 30 s) and extension (72 ºC, 30 s) were carried out in the Mastercycler® epgradient S realplex thermocycler (Eppendorf; Hamburg, Germany) for all genes. The transcripts expression data were normalized to the housekeeping gene *Glyceraldehyde-3-phosphate-dehydrogenase* (*GAPDH*) and the quantification performed according to the Livak method ($2^{-\Delta\Delta Ct}$ method), considering the standard osteogenic differentiation medium (TCPS_osteo) as calibrator.

5.2.5 Statistical Analysis

Data were statistically analyzed using IBM SPSS software (version 20; SPSS Inc.). We first applied the Shapiro-Wilk test to test the assumption of normality and the results showed that the data was not following a normal distribution. Consequently, the non-parametric Mann-Whitney U and Kruskal-Wallis tests were applied. *P* values lower than 0.01 were considered statistically significant in the analysis of the results.

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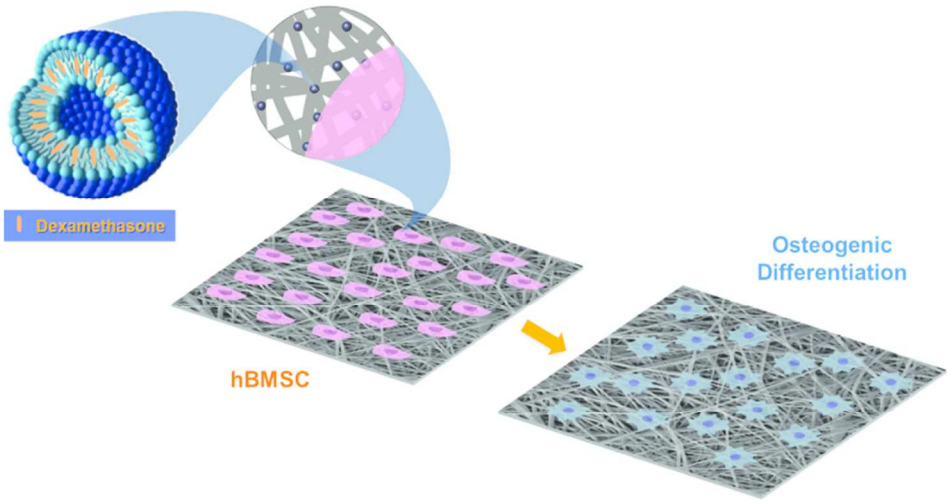

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