Dynamic piezoelectric stimulation enhances osteogenic differentiation of human adipose stem cells

Clarisse Ribeiro,[1,2] Jenita Pärssinen,[3,4] Vítor Sencadas,[1,5] Vítor Correia,[1,2,6] S. Miettinen,[3] Vesa P. Hytönen,[3,4] Senentxu Lanceros-Méndez[1,2,*]

[1] *Center/Department of Physics, University of Minho, Braga 4710-057, Portugal*

[2] *INL - International Iberian Nanotechnology Laboratory, 4715-330 Braga, Portugal*

[3] *BioMediTech, University of Tampere, Tampere 33014, Finland*

[4] *Fimlab Laboratories Ltd., Tampere 33520, Finland*

[5] *Instituto Politécnico do Cávado e do Ave, Campus do IPCA, Barcelos 4750-810, Portugal*

[6] *Algoritmi Research Centre, Universidade do Minho, Campus de Azurém, 4800-058 Guimarães, Portugal.*

*Corresponding author. Address: Center/Department of Physics, University of Minho, Braga 4710-057, Portugal. Tel.: +351 253604073.

E-mail address: lanceros@fisica.uminho.pt

Abstract

This work reports on the influence of the substrate polarization of electroactive β-PVDF on human adipose stem cells (hASCs) differentiation under static and dynamic conditions. hASCs were cultured on different β-PVDF surfaces (non-poled and "poled -") adsorbed with fibronectin and osteogenic differentiation was determined using a quantitative alkaline phosphatase assay. "Poled -" β-PVDF samples promote higher osteogenic differentiation, which is even higher under dynamic conditions. It is thus demonstrated that electroactive membranes can provide the necessary electromechanical stimuli for the differentiation of specific cells and therefore will support the design of suitable tissue engineering strategies, such as bone tissue engineering.

Keywords: Electroactive polymer, stem cell, osteogenesis, dynamic conditions.

1. Introduction

Tissue engineering and regenerative medicine is a multidisciplinary research field that includes different approaches (cells and/or biomaterial) but typically involves a biocompatible biomaterial, which can be combined with stem cells and different stimuli with the objective of repairing failing organs.

Bone regeneration is one of the most promising investigated therapeutic applications in tissue engineering field.[1] Human adipose stem cells (hASCs) exhibit large potential for regenerative medicine applications since they are multipotent and have a natural ability to differentiate into different tissues, such as bone, cartilage and fat cells.[2] In order to induce the differentiation of stem cells to the desired lineage, hASCs require appropriate extracellular stimuli, through chemical (such as growth factors) and physical signals

(such as mechanical stimulation).[3] Particularly, those physical stimuli are of great importance since on an *in vivo* environment cells are continuously subjected to physical stimuli, such as mechanical one, that deeply influence their development. Thus, a mechanical stimulus can increase the activity of specific cells cultured *in vitro* in a similar way as observed for *in vivo* environment.[4-5] In this sense, the use of bioreactors that mimic different stimuli occurring in the body, providing specific biochemical and physical signals can effectively support regulation of cell function.[6-7]

Further, adhesion, proliferation and differentiation of specific cells can be promoted and/or improved by the use of active materials as scaffolds. Recent research has shown the particularly large potential of one class of such active materials: electroactive polymers and, in particular, piezoelectric polymers, as their mechanically varying surface polarization state can influence cell morphology, adhesion, proliferation and differentiation.[8] This fact is particularly important as many body tissues are subjected to varying electro-mechanical solicitation, such as bone,[9] which is fact is piezoelectric, similar to observed for collagen (matrix).[10-11] Thus, varying surface charge can stimulate cell response,[7,12] and the use of piezoelectric polymers as scaffolds may allow to mimic existing mechanical and electrical signals relevant for biological activity. In this way, electroactive polymers emerged as a novel approach for tissue engineering applications. Poly(vinylidene fluoride) (PVDF) is a biocompatible polymer with the largest piezoelectric response known among polymer materials.[13] Although many studies have been performed with PVDF, only a few studies have reported its value as biomaterial. Previous works have shown that piezoelectric polymers are able to induce transient surface charge and they have found to induce a higher cell growth and differentiation compared with the non-piezoelectric control materials.[7,14] It was also demonstrated that when tissue recombinants were cultivated on PVDF, the serum-

deprived effect could be rescued and submandibular gland tissue recombinant was able to increase epithelial size, synthesize basement membrane and develop new branches without serum.[15] Additionally, the human neural stem/progenitor cells (hNSCs/NPCs) differentiation on piezoelectric poly(vinylidene fluoride)-trifluoroethylene (PVDF-TrFE) fibrous scaffolds has shown that contact guidance combined with the piezoelectric properties promote the neurite extension and neuronal differentiation.[16] Further, stimulation under dynamic conditions was already tested showing that it can strongly increase cell response, such as cell proliferation.[7]

Considering the high potential of piezoelectric polymers in development of innovative and new "smart" materials, the challenge lies in the exploitation of the electrical stimuli through the mechanical stimulus. It would be important to proof that these stimuli are relevant for tissue engineering strategies.

In this sense, the aim of this work is to study of influence of electro-mechanical stimulus on osteogenic differentiation. Since the absorption of fibronectin helps cell adhesion,[17] experiments with hASCs cultured on PVDF coated with fibronectin were performed under both static and dynamic conditions, with and without osteogenic medium in order to evaluate the suitability of piezoelectric polymers as smart scaffolds. This work opens new horizons for the use of piezoelectric stimulation in tissue engineering.

2. Experimental Section

2.1 PVDF samples

PVDF films (110 μm of thickness) were prepared by spreading a solution of PVDF (Solef 1010, Solvay) in N,N-dimethyl formamide (DMF) (20 wt% PVDF) onto a glass substrate as described previously in [17].

The electrical poling of the β-PVDF films was achieved using a corona discharge inside a home-made chamber at 100 ºC. The applied voltage was 10 kV with a constant current of 15 μA, and the distance between the sample and the tip was 2 cm. Thereafter, the piezoelectric response (*d*33) of the poled samples was verified with a wide range *d*33-meter (model 8000, APC Int. Ltd., Mackeyville, USA). The obtained piezoelectric *d33* coefficient was ~ -32 pC N^{-1}.

The PVDF films used were non-poled β-PVDF and "poled -" β-PVDF (cells cultured on the negatively charged side of the material).

2.2 Samples sterilization

For the *in vitro* assays, circular PVDF films were cut with 13 mm of diameter. The films were sterilized by several immersions in 70% ethanol for 30 min each. After that, the samples were exposed to ultraviolet light (UV) for 1 h.

2.3 Fibronectin adsorption

The fibronectin (FN) coating was performed without and with photoinitiator treatment. The photoinitiator treatment was used in order to introduce covalent coupling between PVDF and deposited fibronectin to ensure, that manipulation of the material does not release the fibronectin. For FN coating (without photoinitiator), FN was adsorbed on the different PVDF films by immersing the material sheets in FN solution with a concentration of 50 μg mL^{-1} for 30 min.

For covalent coupling with photoinitiator, a photoinitiator solution (benzophenone in absolute ethanol solution) with a concentration of 10 mg mL^{-1} was added to the PVDF samples for 5 min at room temperature. After that, the photoinitiator solution was

removed and the samples were allowed to dry for 1 h in laminar flow at room temperature. Then, the FN solution (50 μg mL^{-1} for 30 min) was added to the PVDF samples. Thereafter, the FN solution was removed and the samples were placed in UV irradiator (IBI Ultralinker, Kodak) for 20 min.

For both fibronectin adsorption processes all samples were washed three times with phosphate buffer saline (PBS) solution before cell culture.

2.4 Cell culture

The adipose tissue samples were collected in accordance with the Ethics Committee of the Pirkanmaa Hospital District, Tampere, Finland (R03058). hASCs were isolated from subcutaneous adipose tissue samples acquired from a surgical procedure performed at the Department of Plastic Surgery, Tampere University Hospital, Tampere, Finland. The hASCs were isolated from the adipose tissue samples of two different patients using the mechanical and enzymatic method described previously in [18]. hASCs were maintained and expanded in maintenance medium consisting of Dulbecco's modified Eagle's medium/Ham's nutrient mixture F-12 (DMEM/F-12 1:1, Invitrogen) supplemented with 1% L-alanyl-L-glutamine (GlutaMAX, Invitrogen), 1% antibiotics (100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin, Invitrogen), and 10% allogeneic human serum (HS, PAA Laboratories GmbH.). The experiments were performed at passages 2 to 4.

Circular PVDF samples were placed in a 24-well tissue culture polystyrene plate and 500 µL of cell supension (4 x 10^3 cell mL^{-1}) was added to each well and incubated at 37 ºC in 95% humidified air containing 5% CO_2. Also, after 7 days of static culture, part of the cell-cultured samples were transferred onto a home-made bioreactor system (dynamic culture).

The dynamic culture was performed with a mechanical stimulation by placing the culture plate on a vertical vibration module at a frequency of 1 Hz with maximum amplitude of ~ 1 mm.

2.5 Quantification of alkaline phosphatase and DNA

According to the procedure described in [18], the *in vitro* osteogenic differentiation capacity was determined 15 days after the initiation of differentiation using the alkaline phosphatase quantification assay (qALP).

Briefly, the cells were cultured in regular maintenance medium as well as in osteogenic medium (the cell differentiation was started after 24 h and the maintenance medium was supplemented with 5 nM dexamethasone (Dex, Sigma-Aldrich), 250 μM L-ascorbic acid 2-phosphate (AsA2-P, Sigma-Aldrich) and 10 mM β-glycerophosphate (β-GP, Sigma-Aldrich)).

To lyse the cells, the cells were collected after 15 days and placed in 0.1% of triton buffer solution (Sigma-Aldrich) and frozen at -70 ºC. Afterward, as described in [18], the amount of p-NP (p-nitrophenol) produced was measured using a microplate reader (BioRad Lab) by recording the absorbance at 405 nm.

To normalize the qALP activity results, the total was quantified from the cell lysate using a CyQUANT Cell Proliferation Assay Kit (Life Technologies Ltd.) in accordance with the manufacturer's protocol. Then, the fluorescence of each sample was measured by exciting the sample at 480 nm and measuring the emission at 520 nm using a BioRad Lab reader.

2.6 Statistical analysis

The quantitative results were obtained from triplicate samples. Statistical differences were obtained by ANOVA using Fisher test for the evaluation of the different groups. P values < 0.005 were considered to be statistically significant. The results are expressed as the mean ± SD (standard deviation).

3. Results and discussion

In our previous studies, the influence of piezoelectric PVDF films on fibronectin adsorption[17] and osteogenic differentiation under static conditions[18] showed that the surface charge of the poled β-PVDF films influence the conformation of adsorbed fibronectin which modulated the hASCs adhesion on the PVDF films and induced their osteogenic differentiation. It was also verified that the different types of β-PVDF (non-poled, "poled +" and "poled -") affect in a different way cell adhesion, proliferation and differentiation and that the influence is dependent on cell type. In particular, investigations with hASCs cultured on PVDF films[18] has shown that the cell adhesion on "poled -" β-PVDF samples is stronger than in cells cultured on the other types of PVDF films. For that reason, "poled -" β-PVDF was used here to study the potential of piezoelectric stimulation to affect cellular differentiation (Figure 1). Non-poled β-PVDF was used as control material without piezoelectric function.

Figure 2 shows the hASCs differentiation on different PVDF films determined by the relative qALP expression after 15 days of culture using regular and osteogenic medium under static and dynamic conditions. Regarding static and dynamic conditions, it is verified a higher osteogenic differentiation on "poled -" β-PVDF in dynamic conditions

than in static conditions. This behavior was not observed for the non-poled β-PVDF samples. Although osteogenic medium enhanced differentiation as compared to regular medium in all the conditions studied, piezoelectric stimulation showed further enhancement in differentiation when cells were cultured on "poled -" β-PVDF but not when cultured on non-poled PVDF. The highest amount of differentiation was obtained by combining piezoelectric stimulation and chemical stimulation, i.e. when cells were cultured on "poled -" β-PVDF in osteogenic medium and under dynamic conditions.

Control experiment where fibronectin was crosslinked to PVDF by using benzophenone crosslinker was also performed and it was observed virtually identical results as compared to those seen in case of fibronectin physisorption. This finding suggests that fibronectin physisorption to PVDF is tight enough to resist piezoelectric stimulation procedure and the effect cannot be further enhanced by covalent linking of the protein layer. In this context, it is good to mention that fibronectin can be considered as “sticky” protein, and the importance of covalent coupling may vary between proteins.

In conclusion, it is possible to claim that the incorporation of both physical and chemical stimulus in *in vitro* culture enhances the osteogenic differentiation. More particularly, piezoelectric materials may provide the necessary electrical stimulus for osteogenic differentiation, mimicking the mechanically stimulated environments existing in the body, improving thus bone regeneration strategies.

4. Conclusions

This work proves that the use of a suitable combination of physical and biochemical stimuli in a biomimetic approach can lead to the development of more efficient and successful tissue engineering strategies. In particular, the use of piezoelectric stimuli,

mechanically induced variations of surface charge, can lead to enhanced osteogenic differentiation of human adipose stem cells (hASCs). Therefore, dynamic mechanical conditions in combination of suitable osteogenic differentiation media may offer tools to better mimick the conditions found *in vivo*.

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Figures and Figure Captions

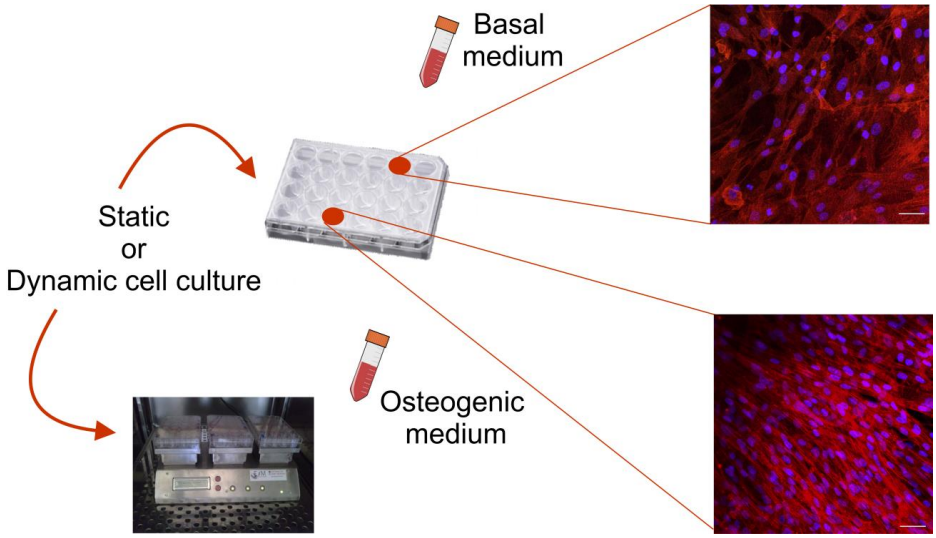

Figure 1 - Scheme of cell culture assay (static and dynamic conditions; basal and osteogenic medium) and representative confocal images of cells cultured 7 days on "poled -" β-PVDF samples. The confocal images are presented in the same scale (scale bar of 50 µm).

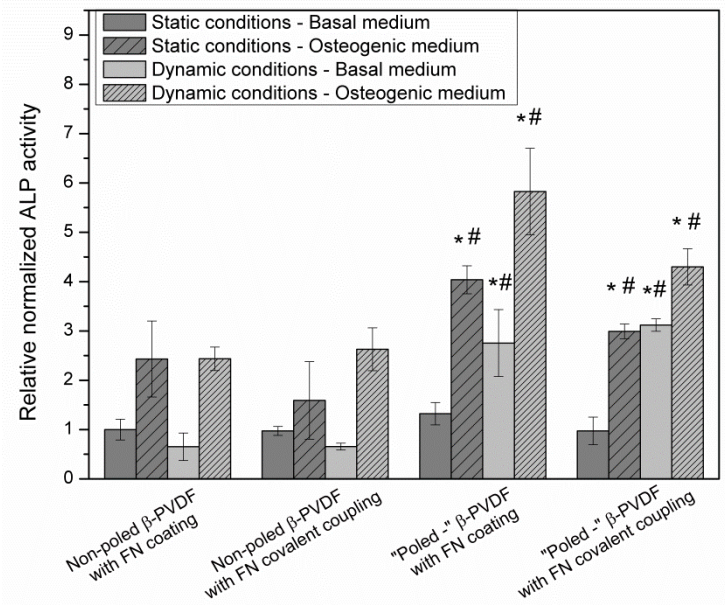

Figure 2 - hASCs differentiation on different PVDF films determined by relative qALP expression after 15 days of culture using regular and osteogenic medium under static and dynamic conditions. The ALP expression was normalized against the DNA content of the cells using the CyQuant cell proliferation assay. * $P \leq 0.005$ vs non-poled β-PVDF with FN coating under static conditions and basal medium; # $P \leq 0.005$ vs "poled -" β-PVDF with FN coating under static conditions and basal medium.