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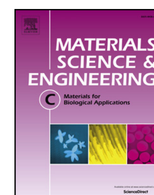

Electroactive biomaterial surface engineering effects on muscle cells differentiation

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A B S T R A C T

Even though skeletal muscle cells can naturally regenerate as a response to insignificant tissue damages, more severe injuries can cause irreversible loss of muscle cells mass and/or function. Until now, cell therapies are not a good approach to treat those injuries. Biomaterials such as poly(vinylidene fluoride), PVDF, can improve muscle regeneration by presenting physical cues to muscle cells that mimic the natural regeneration environment. In this way, the ferroelectric and piezoelectric properties of PVDF offer new opportunities for skeletal muscle tissue engineering once the piezoelectricity is an electromechanical effect that can be used to provide electrical signals to the cells, upon mechanical solicitations, similar to the ones found in several body tissues. Thus, previous to dynamic experiments, it is important to determine how the surface properties of the material, both in terms of the poling state (positive or negative net surface charge) and of the morphology (films or fibers) influence myoblast differentiation. It was observed that PVDF promotes myogenic differentiation of C2C12 cells as evidenced by quantitative analysis of myotube fusion, maturation index, length, diameter and number. Charged surfaces improve the fusion of muscle cells into differentiated myotubes, as demonstrated by fusion and maturation index values higher than the control samples. Finally, the use of random and oriented β-PVDF electrospun fibers scaffolds has revealed differences in cell morphology. Contrary to the randomly oriented fibers, oriented PVDF electrospun fibers have promoted the alignment of the cells. It is thus demonstrated that the use of this electroactive polymer represents a suitable approach for the development of electroactive microenvironments for effective muscle tissue engineering.

1. Introduction

The loss or failure of an organ or tissue is one of the most frequent, devastating and costly problems in health care. Musculoskeletal conditions are the most common cause of severe long-term pain and physical disability [1], and they affect hundreds of millions of people around the world [2]. The musculoskeletal system, which includes bone [3], cartilage [4], tendon/ligament [5] and skeletal muscle [6], is becoming the main target for tissue engineering because of the high need for regeneration and/or repair.

Skeletal muscles, comprising between 40 and 45% of an adult human body mass, are mainly responsible for generating forces which facilitate voluntary movement, postural support, breathing and locomotion [7]. Skeletal muscle injuries can stem from a variety of events, including direct trauma such as muscle lacerations, contusions or strains, and indirect causes, such as ischemia, infection or neurological dysfunction [8]. In response to minor injuries, skeletal muscle possesses a remarkable robust innate capacity for regeneration [9]. However, severe injuries that result in muscle mass loss of more than 20% can lead to extensive and irreversible fibrosis, scaring and loss of muscle function [10]. In such cases, reparative fibrosis overpowers formation of new muscle, leaving an excess of scar tissue that eventually yields a sub-innervated, malfunctioning muscle. Furthermore, with aging and severe congenital disorders, the loss of muscle mass and function is additionally exacerbated by the reduced self-renewing capacity of satellite cells [9].

In this sense, tissue engineering and regenerative medicine are growing fields of interest in human life sciences due to promising results in regenerating, maintaining or improving almost every tissue function of the human body by combining optimized biomaterial

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scaffolds, cells and growth factors [11]. The biomaterial scaffold acts as a tool to locally control and guide tissue regeneration, more specifically, its architecture and physical properties play an important role from the cell seeding procedure to the processes of cell proliferation and differentiation [12]. In the human body, various biophysical and biochemical stimuli govern the development and maintenance of tissue structure and functionality. Chemical, mechanical, topography/microstructure, electric and magnetic stimuli are critical cues in the formation, function and regeneration of tissues and organs [13–15]. In this sense, *in vivo* electric signals directly influence the development, function and repair of many tissues and organs. Skeletal muscle cells develop endogenous electrical fields in the form of membrane potentials, which demonstrate the importance of the application and modulation of electrical stimuli [16]. Muscle tissue consists of aligned bundles of multinucleated, striated and contractile muscle cells, the myofibers [17]. It has been demonstrated that electrical stimulation not only influences muscle cell phenotype, myosin expression and contractile sarcomere assembly, but can also modulate fiber type switch, and induce contractility in differentiated myotubes [18]. C2C12 myoblasts, a murine myoblast cell line, is routinely used as an experimental model of skeletal muscle [6]. Myotubes differentiated from C2C12 myoblasts can be used to monitor skeletal muscle cell contraction and activity [19,20]. It has been demonstrated that Ca^{2+} transient fluxes in C2C12 myotubes induced by electrical pulse stimulation can accelerate the combination of functional sarcomeres and stimulate contractile activity of cells [21]. Furthermore, C2C12 skeletal muscle cells cultured on positively and negatively charged polyelectrolyte layer-by-layer polymeric nanofilms demonstrate that negative charge and the increase of the molecular weight promote myotube formation with high fusion index [22].

As electrical signals, in particular electromechanical signals, constitute one of the main physical stimuli present in the human body, electroactive scaffolds based on piezoelectric polymers have shown strong potential for tissue engineering [3,23] with different cellular models, such as C2C12 myoblast [6], MC3T3-E1 pre-osteoblast [24,25], human adipose stem cells [3] and also in *in vivo* study [26]. Piezoelectric polymers are able to induce transient surface charge boosting cell growth and differentiation compared with non-piezoelectric controls. In this way, such electromechanical stimulation can be conducted effectively by piezoelectric polymers, such as poly(vinylidene fluoride) (PVDF) that is a biocompatible polymer with the largest piezoelectric response. PVDF is a semi-crystalline polymer with four known crystalline forms: β, α, γ and δ, the β-phase showing the strongest piezoelectric response [27,28]. Further, materials with a magnetoelectric response based on the combination of magnetostrictive and piezoelectric materials have been proposed as a new approach for tissue engineering [29,30], allowing magnetically induced piezoelectric stimulation of cells.

In this context, the main objective of the present study is to elucidate the influence of the poling state in the behavior of C2C12 myoblast cell line cultured on β-PVDF films, quantifying the differentiation capabilities of myoblasts, such as the fusion and maturation index. Three kinds of β-PVDF films were used: "Non-poled" - no charged surface, "poled +" - positively charged side of the sample and "poled −" – negatively charged side of the sample. Oriented and random electrospun fibers were used to evaluate the effect of piezoelectric support morphology on cell response. C2C12 differentiation into myotubes was analyzed in order to provide quantitative information on the regeneration potential of skeletal muscle through piezoelectric polymers.

2. Materials and methods

2.1. Materials

PVDF (Solef 5130, M_W 1000–1200 kg/mol) and *N*,*N*-dimethylformamide (DMF) were purchased from Solvay and Merck, respectively. A 20% (w/w) solution of PVDF in DMF was prepared under magnetic stirring at room temperature until complete dissolution of the polymer.

2.2. Preparation of the samples

For PVDF films, the solution was spread on a clean glass substrate and heated (J.P. Selecta) to 220 °C for 10 min for solvent evaporation and polymer melting. Then the samples were cooled at room temperature. After that, the polymer is predominantly in the α-PVDF, so to obtain β-PVDF, the conventional stretching is carried out and films with a thickness around 110 μm were obtained [31]. Sample poling (orientation of the dipolar moments along the thickness direction of the samples) was achieved by Corona discharge inside a home-made chamber at 10 kV and 10 μA, after an optimization procedure [31]. The piezoelectric d_{33} coefficient of the poled samples is ≈ -32 pC·N^{-1} [27].

The PVDF electrospun fibers were processed after the experimental procedure presented in [31,32]. In short, the polymer solution was placed in a plastic syringe (10 mL) fitted with a steel needle with an inner diameter of 0.5 mm. Electrospinning was conducted with a high voltage power supply from Glasman (model PS/FC30P04) at 14 kV with a feed rate of 0.5 mL·h^{-1} (syringe pump from Syringepump). The electrospun fibers were collected in an aluminum plate placed at 20 cm from the needle and in a rotating drum (1500 rpm), to obtain random and oriented nanofibers, respectively.

The surface charge and morphological features of the films and fibers has been previously reported in [32,33], respectively. Contact angle, essential for properly understanding cell materials interaction, is 83.1° ± 2.2; 51.3° ± 3.1 and 45.0° ± 1.6 for the non-poled, positive poled and negative poled PVDF films, respectively [6]. Further, the contact angle is 135.1° ± 3.0 and 115.6° ± 3.3 for oriented and random fibers, respectively [6].

2.3. Membrane sterilization

Circular PVDF samples for *in-vitro* assays: non-poled, poled "+", poled "−" β-PVDF films, random and oriented β-PVDF nanofibers, were cut with 13 mm of diameter. The samples were sterilized by multiple immersions into 70% ethanol for 30 min each and washed 5 times in a phosphate buffered saline (PBS) solution for 5 min each to remove any residual solvent. Subsequently, the samples were exposed to ultraviolet (UV) light for 2 h (1 h each side). Then, the samples were placed in a 24-well cell culture plates.

2.4. Cell culture

C2C12 cells were grown in 75 cm^2 cell-culture flask and cultured in basal medium (BM), consisting of Dulbecco's Modified Eagle's Medium (DMEM, *Gibco*) containing 4.5 g·L^{-1} supplemented with 10% Fetal Bovine Serum (FBS, *Biochrom*) and 1% Penicillin-Streptomycin (P/S, *Biochrom*). The flask was incubated at 37 °C in a humidified air containing 5% CO_2 atmosphere. The culture medium was changed every two days and when the cells reached 60–70% confluence, they were trypsinized with 0.05% trypsin-EDTA. C2C12 cells were seeded on the different PVDF films and fibers at a density of 4×10^4 cells·mL^{-1} and 1×10^5 cells·mL^{-1}, respectively. The samples were placed in 24-well plates and incubated in BM, as previously described, until becoming confluent. For proliferation assessment, the cells were maintained in BM for 8 days at 37 °C in a saturated humidity atmosphere containing 95% air and 5% CO_2. For the differentiation experiments, in order to induce myotube formation, the BM of the C2C12 cells was changed to differentiation medium (DM) and the culture was also maintained for 8 days, as in the proliferation studies. The DM was composed of DMEM supplemented with 2% FBS and 1% P/S. The decrease of the serum percentage in the medium (DM) leads the cells to be exposed to less

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