PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE

ESCUELA DE INGENIERIA

BIOCOMPATIBILITY STUDIES OF CHITOSAN/POLY(OTE) BLENDS AS A SCAFFOLD TO MAINTAIN MYOBLASTS REGENERATION POTENTIAL IN VITRO

CRISTINA A. PADILLA

Thesis submitted to the Office of Research and Graduate Studies in partial fulfillment of the requirements for the Degree of Master of Science in Engineering

Advisors:

LORETO M. VALENZUELA

HUGO C. OLGUIN

Santiago de Chile, (June, 2016)

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To my parents

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LIST OF ABREVIATIONS

AFM	Atomic Force Microscopy
BCA	Bicinchoninic acid assay
CP	Conducting polymers
DAPI	4,6-diamidino-2-phenylindole
DD	Degree of deacetylation
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco´s modified eagle medium
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FGF	Fibroblast growth Factor
FTIR	Fourier Transform Infrared Analysis
GAGs	Glycosaminoglycans
HA	Hyaluronic acid
HGF	Hepatocyte Growth Factor
HRP	Horseradish peroxidase
HSKM5	Human adult skeletal muscle cell line
HMbs	Human primary myoblasts
hSKMCs	Human skeletal muscle cell
H&E	Hematoxylin- Eosin
IGF	Insulin-like growth factor
IL	Interleukin
MHC	Myosin heavy chain
MSC	Mesenchymal stem cell
MPC	Muscle progenitor cell
NMR	Nuclear magnetic resonance

PANI	Polyaniline
PBS	Phosphate buffered saline
PCL	Polycaprolactone
PDLA	Poly(D-lactic acid)
PDLLA	Poly(D,L-lactic acid)
PFA	Paraformaldehyde
PGA	Polyglycolic acid
PLA	Polylactide acid
PLCL	Poly(L-lactide-co-caprolactone)
PLGA	Polylactide-co-glycolic acid
PLLA	Poly(L-lactic acid)
pMbs	Adult mouse primary myoblasts
Poly(OTE)	Poly octanoic acid 2-thiophen-3-yl- ethyl ester
PPy	Polypyrrole
PS	Polystyrene
PTh	Polythiophene
PVDF	Polyvinylidene fluoride
RIPA	Radioimmunoprecipitation assay buffer
SCs	Satellite cell
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
TFA	Trifluoroacetic acid
TNF	Tumor necrosis factor
VEGF	Vascular endothelial growth factor
VML	Volumetric muscle loss

RESUMEN

El desarrollo y utilización de matrices poliméricas como soporte para la entrega de precursores miogénicos es una alternativa prometedora para la ingeniería de tejido muscular esquelético. Estas matrices tienen como propósito actuar como sustitutos temporales del nicho miogénico ya sea *in vivo* o *in vitro,* proporcionando los estímulos necesarios para la supervivencia celular, proliferación y eventualmente la formación de músculo esquelético. Estudios previos en el laboratorio utilizando películas de quitosano como potencial matriz polimérica mostraron una baja adhesión de células C2C12 y mioblastos primarios de ratón (pMbs). Con el fin de mejorar la adhesión y biocompatibilidad de estas células, decidimos explorar el uso de quitosano en mezcla con el polímero conductor poli-(ácido octanoico 2-tiofen-3-il-etil éster) (poli(OTE)). Con este propósito, obtuvimos y caracterizamos películas de quitosano y mezclas quitosano/poli(OTE) con 3% y 6% p/v de poli(OTE), para determinar la respuesta celular *in vitro* de mioblastos de ratón. Los resultados obtenidos mostraron la formación películas en las que poli(OTE) se encontraba disperso en quitosano formando dominios, con un aumento en la rugosidad de superficie de la película al aumentar la concentración de poli(OTE). Estas mezclas mejoraron el numero de células adheridas en C2C12 y pMbs al comparar con películas de quitosano puro, sin estimular su diferenciación. De hecho, estas células no fueron capaces de diferenciarse en estas mezclas, pero mantuvieron su compromiso miogénico y la capacidad de diferenciarse. Estos resultados sugieren que las mezclas quitosano/poli(OTE) podrían ser un sustrato o matriz apropiada para la mantención del potencial regenerativo de mioblastos *in vitro*, retardando el proceso de diferenciación previo al transplante, lo cual es un problema limitante en medicina regenerativa. Además se determinó que C2C12 podría ser un mejor modelo celular que pMbs para estudios *in vitro* con la mezcla quitosano/poli(OTE).

Palabras Claves: Quitosano, poli(OTE), C2C12, mioblastos, Pax7, regeneración músculo esquelético.

ABSTRACT

The delivery of satellite cells/myoblasts using engineered polymer scaffolds is a promising approach to achieve skeletal muscle regeneration. These scaffolds intend to act as temporarily surrogate for myoblasts niche either *in vitro* or *in vivo,* providing needs for cell survival, proliferation and eventually tissue formation. Previous studies in our laboratory with chitosan films as a potential polymer scaffold showed poor myoblast adhesion of C2C12 and mouse primary myoblasts (pMbs). In order to improve myoblast adhesion and biocompatibility on these films, we decided to explore the use of chitosan blended with the conductive polymer poly-(octanoic acid 2-thiophen-3-yl-ethyl ester) (poly(OTE)). We obtained and characterized films of chitosan and chitosan/poly(OTE) blends with 3 and 6% w/v of poly(OTE) to determine their *in vitro* cell response with mouse skeletal muscle progenitors. Our results showed that chitosan/poly(OTE) films presented poly(OTE) domains dispersed in chitosan, with an increase on films roughness with increasing poly(OTE) concentration. These blends improved the number of adherent cells in C2C12 and pMbs when compared with pure chitosan films, without stimulating their differentiation. In fact, these cells were unable to differentiate on the blends but they maintained their lineage commitment and differentiation potential. These results suggest that chitosan/poly(OTE) blends could be a suitable substrate/scaffold to maintain myoblasts regeneration potential *in vitro,* retarding differentiation prior to transplantation, which is a critical issue in regenerative medicine. Also, it was determined that C2C12 cells could be a better cell model than pMbs for future *in vitro* studies on chitosan/poly(OTE) blends.

Keywords: Chitosan, poly(OTE), C2C12, myoblasts, Pax7, skeletal muscle regeneration.

1 SKELETAL MUSCLE BIOLOGY

Skeletal muscle is a highly organized and the most abundant tissue in the human body. It has been estimated that humans have approximately 640 muscles, corresponding to the 30% of body mass in women and 38% in men (Janssen et. al, 2000). The most important functions of this tissue include body posture maintenance, force generation to permit a precise movement of bone and articulations in different parts of the body, protein reservoir, and its participation in energy metabolism (Karagounis & Hawley, 2010).

1.1 Skeletal muscle structure

Each skeletal muscle is connected to the bone by a tendon, which is composed mainly by collagen, forming the myotendinous junction. These junctions transmit force longitudinally along the long axis of the muscle to result in movement. Skeletal muscle is structurally formed by groups of muscle fibers called fascicles that are surrounded by a thin layer of collagen, blood vessels, nerves, among other fibrous proteins from the extracellular matrix (ECM), and polysaccharides. Each muscle fiber or myofiber is a long cylindrical structure that corresponds to the functional unit of skeletal muscle, which can generate force by its contraction. These myofibers are multinucleated structures that may extent the length of an entire muscle. They are comprised by a great number of sarcomeres that are arranged in series along their lengths. Sarcomeres are mainly formed by the contractile proteins actin and myosin and other proteins to maintain their arrangement (McNally et al., 2006; Relaix & Zammit, 2012). A group of cells called satellite cells are located at each myofiber surface; these are quiescent stem cells that participate in the generation of new muscle fibers upon damage. Each muscle fiber with its own pool of satellite cells, is surrounded by a basal lamina (Relaix & Zammit, 2012). Skeletal muscle structure is schematized on Figure 1.

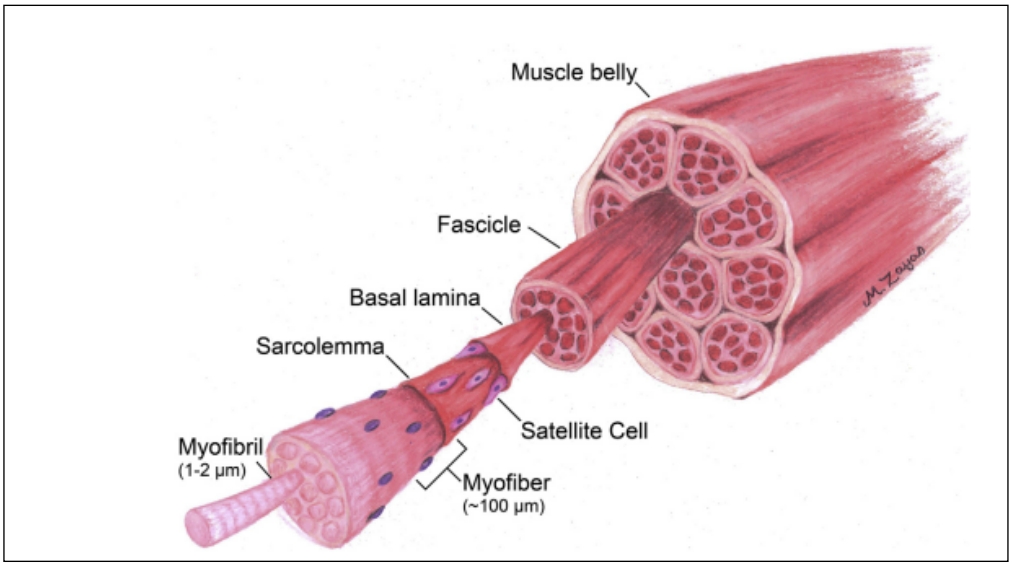

Figure 1. Schematic illustration of skeletal muscle tissue organization. Obtained from Grasman et al. (2015).

1.2 Skeletal muscle contraction

Sarcomeres represent the contractile apparatus of the muscle fiber, formed by an organized unit of thick (myosin) and thin filaments (actin) (Greising & Gransee, 2013; Koffler et al., 2011; McNally et al., 2006). Each sarcomere is anchored and attached to each other by a group of proteins called Z-disc that are arranged perpendicular to the filaments. Z-discs are responsible for the striated appearance, which is one of the main characteristics of skeletal muscle (Figure 2). During muscle contraction, initiated by neural signals, actin filaments are displaced to the center of the sarcomere (M-line), producing an increased overlap between actin and myosin filaments due to the formation of crosslinking bridges, which produces a decrease in the longitudinal length of the sarcomere. The force produced by this process is related to the level of overlapping and the level of crosslinking between these filaments. The force produced by this overlap is transmitted longitudinally to the Z-disc, and then from one sarcomere to the next in series until it achieves the tendon, were the force is transmitted in a passive manner to produce movement (Greising et al., 2013; McNally et al., 2006).

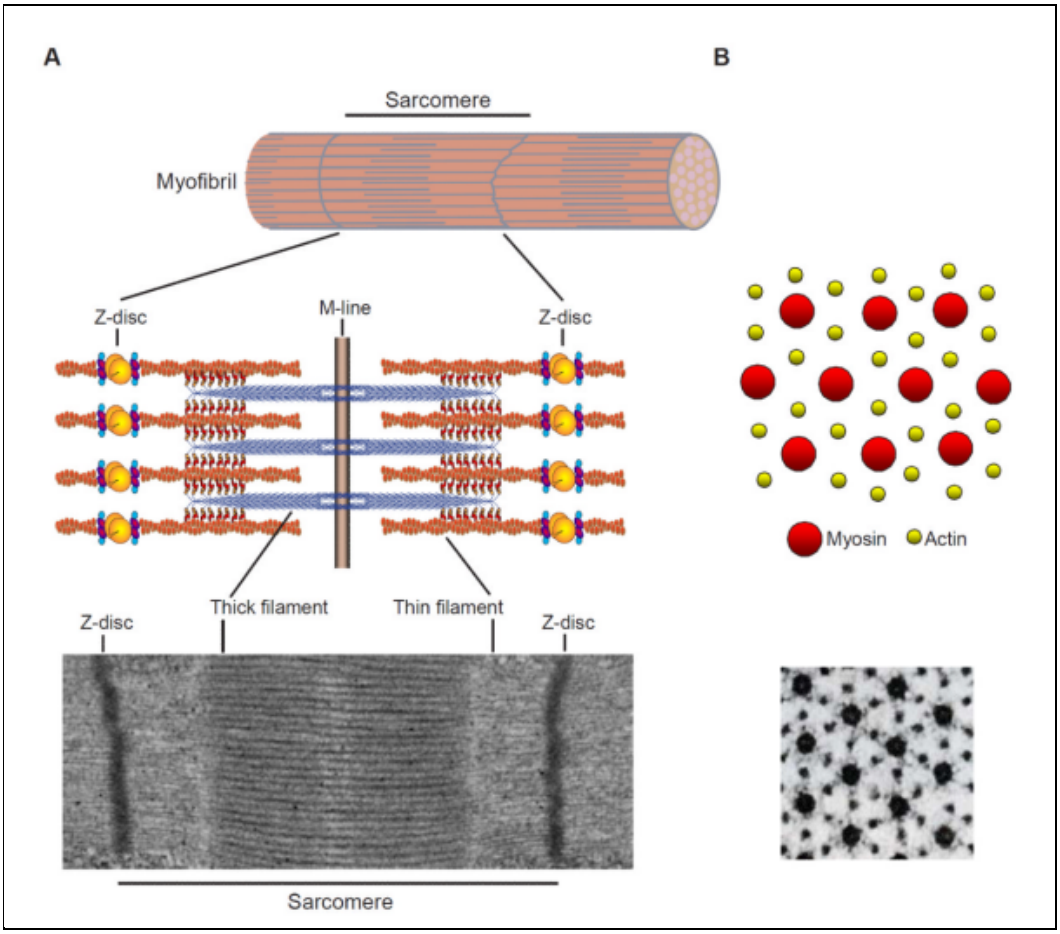

Figure 2. Representation of sarcomeres arrangement.
A. Front section of a sarcomere seen by electron microscopy with a schematic representation on top. **B.** Schematic representation of myosin (red) and actin (yellow) filaments organization with an electron micrograph of a muscle fiber cross-section. Obtained from Greising et. al. (2013).

1.3 Regenerative capacity of skeletal muscle

Skeletal muscle has a very good mechanism to regenerate itself in response to damage by exercise or mechanical injury due to the presence of satellite cells, which function as muscle-specific adult stem cells. In adults, skeletal muscle tissue regeneration occurs mainly in three steps: 1) inflammation, 2) skeletal muscle tissue formation and 3) maturation and remodeling, that can lead to the formation of fibrous or scar tissue when severe damage occurs.

1.3.1 Inflammation

Immediately after muscle damage there is necrosis and digestion of the different components of the damaged tissue by proteases. These events trigger an inflammatory response in the damaged area, characterized by the recruitment of leucocytes, neutrophils and lastly, macrophages. These last two cell types have the most important role in the response of skeletal muscle after damage (Ciciliot & Schiaffino, 2010). Neutrophils are the first inflammatory cells that invade the muscle, within 2 hours of injury, followed by macrophages (Kharraz et al., 2013). Studies have shown that there are two macrophage subpopulations that invade the lesion area consecutively. Initially, macrophages that invade the damaged area during the first 24 hours secrete pro-inflammatory cytokines such as: Tumor Necrosis Factor (TNF) and Interleukin 1 (IL-1), which induce phagocytosis of the damaged tissue. After the first day, pro-inflammatory phenotype macrophages start to decrease in number and anti-inflammatory phenotype increases, which secrete anti-inflammatory cytokines such as Interleukin 10 (IL-10) in order to cease inflammation and stimulate satellite cells activation (Chazaud et al., 2009).

1.3.2 Skeletal muscle formation

Normally satellite cells are in quiescent state in the basal lamina, expressing molecular markers such as the transcription factor Pax-7 (Seale et al., 2000; Zouraq et al., 2013). After damage, different growth factors are secreted in the lesion area: Fibroblast Growth Factor (FGF), Transforming Growth Factor (TGF), Insulin-like Growth Factor I and II (IGF-I, IGF-II), Hepatocyte Growth Factor (HGF) and Interleukin 6 (IL-6). These growth factors, together with other signals such as the liberation of nitric oxide, activate satellite cells and stimulate them to migrate to the lesion area (Grefte et al., 2007; Hawke & Garry, 2001). Activated satellite cells maintain Pax-7 expression, but also induce the expression of muscle regulatory factors MyoD, Myf5 and start to proliferate. In this stage, these cells are called myoblasts. MyoD and Myf5 begin to stimulate myoblast differentiation, inducing myogenin and Mrf4 expression, while Pax-7 levels decrease (Olguin & Olwin,

2004; Zouraq et al., 2013). These changes stimulate myoblasts terminal differentiation, including myoblast fusion in order to form new myofibers or repair damaged myofibers. Molecular markers for terminal differentiation include desmin, α-actinin and myosin heavy chain (MHC) (Zouraq et al., 2013) (Figure 3). On the other hand, satellite cells are capable of self-renewal in order to maintain their pool and maintain the regenerative capacity of the muscle tissue. Because of this characteristic, an asymmetric cell division mechanism is proposed. This mechanism describes two subpopulations of satellite cells: a Pax7+/Myf5+ subpopulation, that correspond to those cells that acquire a myogenic compromise and begin to expand in order to form new myofibers, and a Pax7+/Myf5- subpopulation that consider satellite cells that return to quiescence and rejoin the satellite cell pool (Kuang et al., 2007; Tedesco et al., 2010; Zammit et al., 2004).

1.3.3 Maturation and remodeling

During this last stage, new myofibers are aligned, fused and reorganized in order to restore the muscle tissue structure. Some characteristics of this process are an increase in fiber diameter, and migration of myofibers nuclei into the fiber periphery (Grefte et al., 2007; Järvinen et al., 2005). Muscle is reintegrated to the rest of the tissue by re-vascularization and re-innervation, there is remodeling of ECM and apoptosis of myofibroblasts, that participated during the inflammation and repair stages (Järvinen et al., 2005). In spite that most skeletal muscle tissue regenerates completely restoring its functionality, this is not always the case. For where there is extensive and/or chronic damage there is an excessive myofibroblast proliferation, which entails dense scar tissue formation producing a mechanical barrier that delays or prevents complete muscle regeneration and thus, preventing complete functional restoration of the tissue (Järvinen et al., 2005).

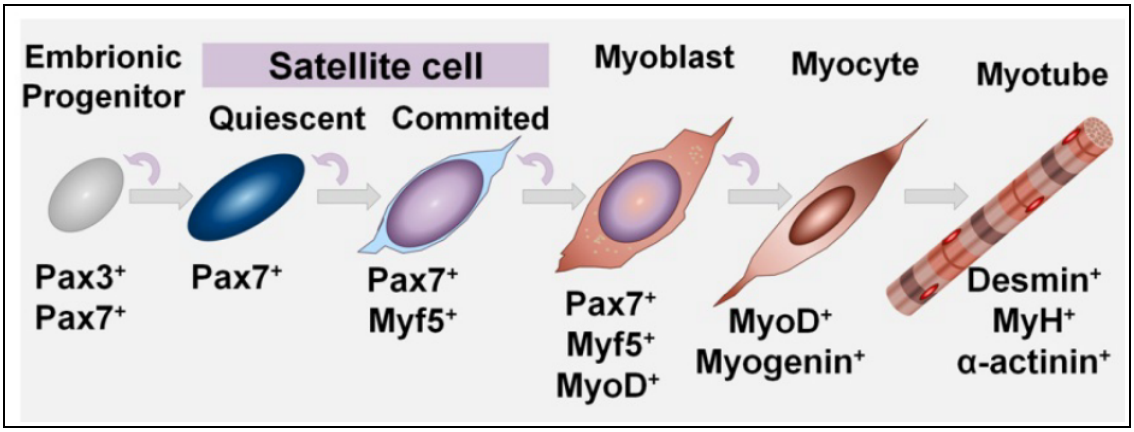

Figure 3. Schematic representation of the myogenic cell lineage.
MyH^{+}: Myosin heavy chain (MHC). Image obtained from Zouraq et al. (2013).

1.4 Clinical implications

In spite of the large regenerative capacity of skeletal muscle, the ability of a muscle to repair itself after damage is dependent on the type and severity of the injury. When more than 20% of the muscle is lost, the natural repair process fails to repair the defect (Turner & Badylak, 2012). Volumetric muscle loss (VML) is defined as the traumatic or surgical loss of skeletal muscle with resultant functional impairment (Grogan & Hsu, 2011). Most common cases of VML result from automobile accidents, military injuries, sport injuries or major surgeries where a large amount of soft tissue needs to be removed (*e.g.,* for tumor removal). In these cases, loss compromises basal lamina and satellite cells. As a consequence, regeneration is aberrant, the remaining muscle mass atrophies and a lot of fibrosis and chronic damage is generated, which leads to irrevocable loss of function of the damaged muscle (Corona et al., 2013)

A similar mechanism occurs in muscular dystrophies, hereditary diseases that are characterized by a progressive muscle mass loss and degeneration. These diseases originate due to lack or defect of different proteins located in the plasma membrane or organelle membranes in cells. Each disease is characterized by the loss of function of a particular protein (Emery, 2002). These defects cause musculature damage during contraction, which leads to myofiber damage and severe inflammation. This triggers repetitive cycles of muscular damage and regeneration, which eventually leads to

chronic damage, characterized by a volume reduction of the native tissue and satellite cell depletion. These events produce massive fibrosis and fat accumulation during each chronic damage cycle (Brunelli & Rovere-Querini, 2008). Duchenne's Muscular Dystrophy (DMD) is the most common muscular dystrophy, with an incidence of 21.7 of 100,000 male births (male-linked disease) (Brunelli & Rovere-Querini, 2008). The gene involved in this disease locates in the X chromosome encoding the protein dystrophin, protein that connects cytoskeletal proteins with membrane proteins and the ECM. Young boys with DMD start to manifest motor problems approximately at the age of 6. From this point forward, muscular damage starts to progress, which makes impossible for them to walk when they reach adolescence. Their lifespan does not exceed the second decade, mainly because of respiratory and cardiovascular problems (G. Q. Wallace & McNally, 2009).

Till date, treatments for VML and muscular dystrophies are scarce. Current treatments include autologous transplant, where skeletal muscle is removed from another region of the body and implanted in the damaged area. The main issue with this treatment is that a complete restoration of the damaged area is not always achieved and, it can damage the donor area with risks such as infection and fibrosis. Due to the downsides of this treatment, there is a need for alternative treatments for this condition. Tissue engineering and regenerative medicine is a promising alternative, and could play a vital role in preventing scar tissue formation and restore muscle function in moderate injuries and in patients with severe muscle loss (Qazi et al., 2015).

2. TISSUE ENGINEERING

Tissue engineering is an interdisciplinary science that combines principles and elements of engineering and biology in order to understand tissue structure-function relationships for the development of biologic substitutes (Nerem & Sambanis, 1995). This science arises as an alternative to the lack of tissues and organs for transplant, giving new possibilities of structural and functional reconstruction.

2.1 Skeletal muscle engineering

Tissue engineering uses the regenerative potential of stem cells in order to generate new tissue or organs. Ideally, these stem cells should be autologous to avoid problems with compatibility and immunogenic rejection. For skeletal muscle engineering, cells should be extracted from the patient, cultured, expanded and capable of differentiating into the cell types of muscle tissue. As reviewed in Fishman et al. (2013), there are different stem cells that have this myogenic potency, such as: mesoangioblasts and pericytes from blood vessels, hematopoietic CD133+ cells, embryonic stem cells (ESC), mesenchymal stem cells (MSC), and satellite cells (SCs), among others. One of the most studied cell type in this area till date are satellite cells, mainly because they can be harvested from muscle tissue, which is easy accessible and cells are already predisposed to myogenic differentiation (Koning et al., 2009). However, their main downsides include a low yield per biopsy and difficulties expanding them and controlling differentiation *in vitro* (Fishman et al., 2013). Therefore, immortalized cell lines are used frequently to do basic skeletal muscle engineering experiments. One of the most used cell line for this purpose is C2C12, a pure population of myogenic cells that proliferate and differentiate rapidly in culture, forming contractile myotubes and synthesizing characteristic muscle proteins (Grabowska, et al., 2011). This may not be a good alternative, because established cell lines approximate myogenesis less closely than primary myoblasts (Bach et al., 2003). Also, till date there is a lack of literature that explores how these cell models behave in the presence of different natural or synthetic polymers, which is key in order to develop new engineering strategies and for taking these strategies on clinical trials.

Tissue engineering strategies for repairing skeletal muscle defects have relied on two classical approaches: *in vitro* or *in vivo* tissue engineering (Figure 4). Following, the main characteristics of each approach are described.

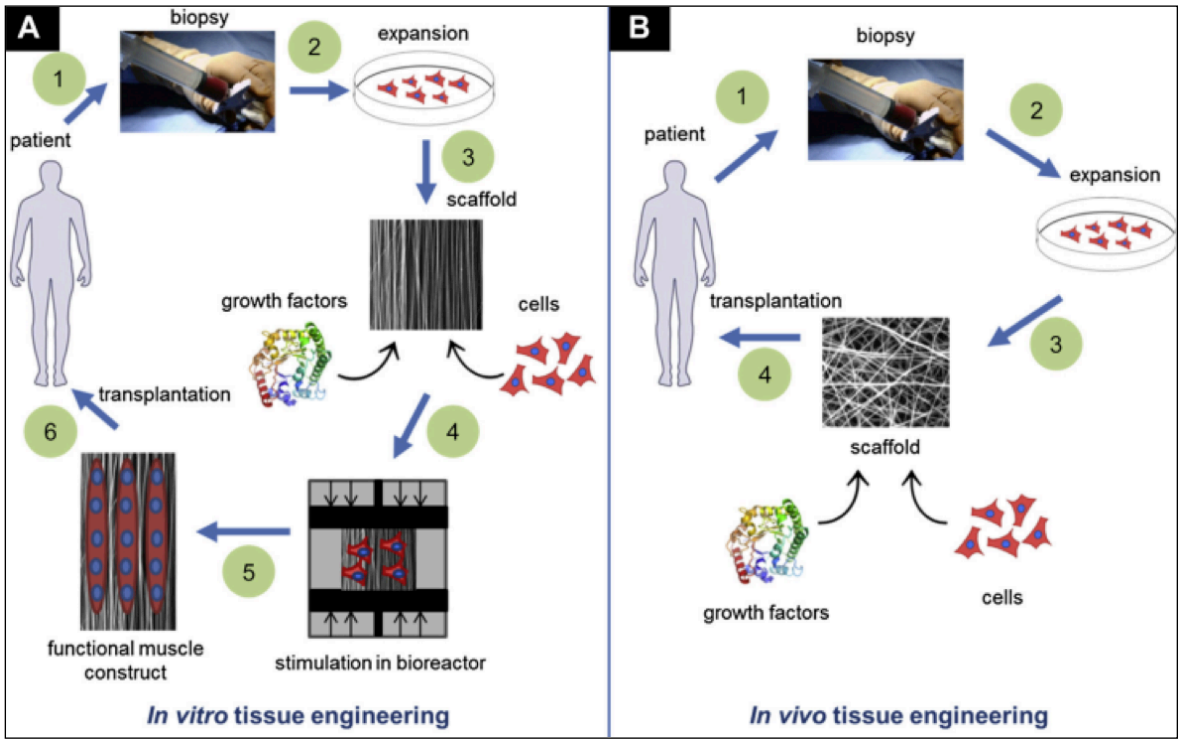

Figure 4. The two classic approaches for skeletal muscle tissue engineering.
A. In vitro tissue engineering involves the development of a functional tissue engineered muscle construct outside the body. **B**. In vivo tissue engineering involves the transplantation of various combinations of cells, growth factors, and scaffolds into the patient. This combination provides a niche from which cells can orchestrate the regeneration process. Image obtained from Qazi et al. (2015).

2.1.1 In vitro tissue engineering

This approach aims creating matured and functional skeletal muscle tissue *in vitro* by culturing autologous cells on a biomaterial scaffold until it has evolved into a functional tissue, which can be transplanted into the patient (Bach et al., 2004; Stern-Straeter et al., 2007) (Figure 4A). Limitations of this approach are ensuring sufficient vascularization to maintain cellular viability in large constructs and

creating engineered tissues that are able to generate physiologically relevant contraction forces (Qazi et al., 2015).

2.1.2 In vivo tissue engineering

This approach consists in a functional reconstruction of skeletal muscle tissue starting from autologous stem cells culture and expansion *in vitro*, obtained from the patient biopsy, followed by re-implantation of these cells in the lesion area using a transport matrix or scaffold that allows their subsequent differentiation *in vivo*, in order to fuse with the remnant tissue (Bach et al., 2004; Stern-Straeter et al., 2007) (Figure 4B). In spite that these cells are capable to reincorporate to the tissue, the main limitation of this strategy is that transplanted cells become vulnerable to immune cells, dramatically reducing cell viability (Qazi et al., 2015).

Independent of the approach that may be used, scaffolds are key elements for tissue engineering because they are meant to provide the appropriate chemical, physical and mechanical properties required for cell survival, proliferation and eventually tissue formation. They intend to act as a temporarily surrogate for stem cell niche either *in vitro* or *in vivo,* playing a crucial role in the regulation of stem cell fate (Shoichet, 2010; Ding et al., 2015).

2.2 Biocompatibility

The term biocompatibility refers to the ability of a material, which is intentionally placed within the body, to be able to perform its intended function without inducing uncontrolled activation of immunological response (Pavithra & Doble, 2008). Therefore, this term includes a series of individual phenomena, influenced by multiple factors such as the type of material, the site of implantation and the genetic pool of the host (William, 2014). In terms of the type of material, not only the chemical composition is relevant, but also its shape, size, roughness, porosity, contact angle and sterilization method.

Biocompatibility is essentially a surface phenomenon, represented by cell–cell, cell–polymer and polymer–protein interactions. When cells are encountered with an implant biomaterial they rarely interact directly with it because of the rapid adsorption of

proteins from blood or serum to the surface of the implant. Therefore, cells interact with these proteins and the composition of the adsorbed layer is a key mediator on cell adhesion and behavior (Wilson et al., 2005). On this work, when we refer to biocompatibility we are mainly referring to *in vitro* biocompatibility, which mainly considers the response of cells on *in vitro* culture when encountered with a material, without considering the extra immunological phenomena occurred when a material is placed *in vivo*.

2.3 Polymer scaffolds

In vivo, skeletal muscle ECM provides myofibers with the architecture that support development, function and regeneration. Thus, skeletal muscle tissue engineering requires a scaffold that mimics this architecture, in order to support proliferation and/or differentiation of myoblasts. This scaffold should be a three-dimensional structure that mimics mechanical and biochemical cues of the native tissue (Koning et al., 2009; Putnam & Mooney, 1996). An ideal scaffold for skeletal muscle engineering should fulfill several characteristics such as: i) high affinity to biological surfaces that promotes ECM formation and vascularization, ii) promote adhesion and alignment of myoblasts, iii) flexibility to allow contraction, iv) biodegradability with non-toxic degradation products, v) sufficient porosity and interconnectivity between pores for nutrient and waste transport (Kamelger et al., 2004; Saxena, 2010), and vi) to be conductive to sustain and enhance muscle's spontaneous electric activity. In order to fulfill these requirements, a variety of materials have been used for skeletal muscle tissue engineering, developing different scaffolds with a variety of physicochemical features and compositions. Polymers are the main material for scaffold construction because their flexibility in chemistry gives rise to materials with great diversity of physical and mechanical properties (Ulery et al., 2011). There is a great amount of polymers with different characteristics that can fit to different classifications. In tissue engineering, polymers can be classified in terms of their origin as natural and synthetic polymers. Among synthetic polymers, polyesters and conducting polymers are the most studied in skeletal muscle engineering. On the next three subsections, these are described considering their main characteristics and experimental findings (Tables 1-3).

2.3.1 Natural polymers

Natural polymers origin from natural sources and provide compositional uniqueness that allow different scaffold-biomolecule and scaffold-cellular interactions (Shoichet, 2010). Because of their origin, several natural polymers are biocompatible and biodegradable, which can mimic structures present in different biological environments such as the ECM (Malafaya et al., 2007). Their main drawbacks are having difficulties in controlling the variability from batch to batch, low mechanical strength and limited processability. Nevertheless, their degradability, biocompatibility and availability -among other advantages- make them attractive candidates for biomedical applications (Malafaya et al., 2007). Natural polymers used in skeletal muscle engineering studies include: collagen, fibrin, hyaluronic acid, alginate and chitosan (Table 1).

a) Collagen

Collagen is the main component of the ECM and the most abundant protein in the human body. It is a structural protein that is present in connective tissues like tendon, cartilage and bone among others (Malafaya et al., 2007; Ulery et al., 2011). Regarding cellular interactions, it participates regulating migration, proliferation and cell survival (Malafaya et al., 2007). For skeletal muscle regeneration purposes, three-dimensional sponge-like scaffolds have demonstrated that myogenic cells are able to proliferate and differentiate *in vivo* and *in vitro* (Kroehne et al., 2008; Ma et al., 2011).

b) Hyaluronic acid

Hyaluronic acid (HA) is a lineal polysaccharide from the glycosaminoglycans (GAGs) family. It is also found in the ECM, and has structural and physiologic roles (Ulery et al., 2011). It is capable of interacting with different cell types by specific cellular membrane receptors inducing tissue repair and modulating inflammatory response (Shoichet, 2010). Because of its low mechanic resistance, HA has been cross-linked with different methods in order to improve its mechanic properties (Ulery et al., 2011). Using this approach *in vitro* biocompatibility assays

using mice skeletal muscle myoblasts showed improvement in cellular adhesion and proliferation (Wang et al., 2009). Also, *in vivo* studies using a honeycomb-like scaffold showed myotubes formation surrounding the implant, as well as formation of fibrovascular tissue on rats (Kamelger et al., 2004).

c) Fibrin

Fibrin is a large crosslinked protein composed by fibrinogen. It is formed naturally during clot formation in the wound healing process, when in the presence of the enzyme thrombin, soluble fibrinogen is converted into insoluble fibrin. A fibrin meshwork is formed, which entraps platelets and other components creating a clot that is stabilized through crosslinking (Malafaya et al., 2007; Ulery et al., 2011). This protein supports cell adhesion and growth, and its physical properties can be tuned by the fibrinogen/thrombin ratio in the design of a tissue-engineered scaffold (Malafaya et al., 2007; Shoichet, 2010). Several *in vivo* studies have been performed demonstrating that a fibrin matrix helps with skeletal muscle regeneration in rats without inducing inflammation or fibrosis (Beier et al., 2006), and that it allows regenerated skeletal muscle vascularization and contraction (Borschel at al, 2006).

d) Alginate

Alginate is a hydrophilic, anionic polysaccharide derived from seaweed, formed by linear chains of β-D-manuronic acid and α-L-guluronic acid (Malafaya et al., 2007; Ulery et al., 2011). Its mechanical properties may vary depending on the ratio of manuronic and guluronic acid. A high content of the later produce more rigid gels, while a higher content in manuronic acid results in weaker gels with low porosity (Malafaya et al., 2007). This polymer presents several disadvantages like low degradation *in vivo*, low mechanic resistance and poor cellular adhesion (Ulery et al., 2011). In order to improve myoblasts biocompatibility with alginate, a peptide sequences (*e.g.,* RGD peptides) have been added to the scaffolds (Hill et al., 2006; Rowley et al., 2002). Hill et al. (2006) demonstrated that incorporating RGD peptides in alginate scaffolds improved viability and cellular migration in

mouse primary myoblasts, however biocompatibility was still poor. Another strategy used to improve myoblast biocompatibility with these scaffolds is the encapsulation of growth factors. For example, Borselli et al. (2011) found that local and sustained release of vascular endothelial growth factor (VEGF) and IGF-1 from macroporous scaffolds, used to transplant and disperse mouse myoblasts, significantly enhanced their engraftment, limited fibrosis, and accelerated the regenerative process (Borselli et al., 2011). Also, Hill et al. (2006) found that the delivery of HGF and FGF-2 from macroporous alginate scaffolds improved cellular viability and migration (Hill et al., 2006).

e) Chitosan

Chitosan is a cationic polysaccharide with variable molecular weight obtained from chitin. Chitin is a linear polysaccharide formed by β(1-4) linked N-acetylglucosamine units that forms the exoskeletons of many arthropods (Ulery et al., 2011). When chitin is N-deacetylated by exposure to a highly basic solution or by enzymatic degradation, the result of this treatment produces chitosan, a copolymer composed by N-acetylglucosamine and N-glucosamine linked by β(1-4) glycosidic bonds (Kim et al., 2008; VandeVord et al., 2002) (Figure 5). Because of this, chitosan has a variable structure that can differ in molecular weight, degree of deacetylation (DD) (ratio between glucosamine and N-acetylglucosamine), and spacing of acetyl units. As a result of these structural differences, the properties of chitosan can also vary (Dornish et al., 2001). Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1,000 kD with a DD from 30% to 95% (Kim et al., 2008).

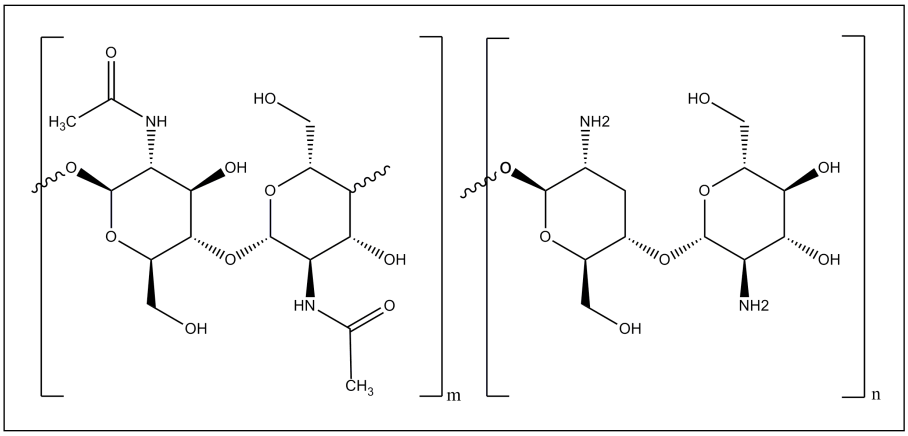

Figure 5. Structure of chitosan.
Copolymer composed by N-acetylglucosamine (m) and N-glucosamine (n) linked by β (1–4) glycosidic bonds.

Chitosan has three reactive functional groups, a primary amine at the C2 position of the glucosamine residues and a primary and secondary hydroxyl groups at C3 and C6, respectively. At low pH amines are protonated, allowing chitosan to be water-soluble. On the other hand, at high pH, amines are deprotonated and chitosan becomes insoluble and uncharged. This soluble-insoluble transition occurs at pH 6-6.5 (Yi et al., 2005).

Chitosan is a semi-crystalline polymer where its degree of crystallinity is dependent on the degree of deacetylation. Crystallinity is maximum for both chitin (i.e., 0% deacetylated) and fully deacetylated chitosan (i.e., 100% deacetylated). Minimun crystallinity is achieved at intermediate degrees of deacetylation (Suh & Matthew, 2000).

Among the main properties that make chitosan a good candidate for biomedical applications are: a chemical structure analogous to GAGs, natural ability to interact with GAGs and proteins because of its cationic nature, and antibacterial and antimicrobial characteristics (Raafat et al., 2008). Finally, chitosan functional groups allow different structural modifications in order to modify chitosan mechanical and biological properties, which could be very useful in tissue engineering (Kim et al., 2008).

Some groups have studied the effect of chitosan in skeletal muscle regeneration using C2C12 or mouse primary myoblasts as cell model. For example, Jana et al. (2013) developed a 3D chitosan scaffold with an appropriate Young's modulus were C2C12 cells readily adhered, proliferated, and differentiated into thick myotubes of approximately 50 μm diameter. Iyer et al. (2013) determined that chitosan upregulated production of integrin β3 in mouse primary myoblasts, possibly for allowing their optimal adhesion and poor adhesion of fibroblasts. Also, several groups have used chitosan blended with synthetic polymers like chitosan/polycaprolactone (Cooper et al., 2010) and chitosan/polyethylenglycol/ gelatin (Hong et al., 2009).

Table 1. Natural polymers studied for scaffolds in skeletal muscle engineering.

Polymer	**Scaffold structure**	**Cell type and technique**	**Results**		**References**
			Proliferation conditions	**Differentiation conditions**	
Collagen	Sponge-like	C2C12 *in vitro* and *in vivo*	Distribution along the scaffold's pores. Ability to proliferate *in vitro*.	Ability to differentiate *in vivo* and *in vitro*	Kroehne et al. (2008)
	Cylindrical and porous	C2C12 *in vitro* and *in vivo*	Distribution along the scaffold's pores. Ability to proliferate *in vitro*	Ability to differentiate *in vivo*	Ma et al. (2011)
Hyaluronic acid	Gel	Rat primary myoblasts *in vitro*	Better proliferation than control	-	Wang et al. (2009)
	Honeycombed	Rat primary myoblasts *in vivo*	-	Ability to differentiate	Kamelger et al. (2004)
	Gel	Fresh isolated mouse CSs *in vivo*	-	Ability to differentiate	Rossi et al. (2011)
Fibrin	Injectable that forms 3D structure *in vivo*	Rat primary myoblasts *in vivo*	-	Ability to differentiate	Beier et al. (2006)
	Cylindrical hidrogel	Rat primary myoblasts *in vivo*	-	Ability to differentiate	Borschel et al. (2006)
Alginate	Nanoporous	pMbs *in vivo*	Low proliferation and viability	-	Hill et al. (2006)
	Macroporous	pMbs *in vitro*	-	Ability to differentiate	Borselli et al. (2011)
Chitosan	3D Uniaxial and porous	C2C12 *in vitro*	Ability to proliferate	Ability to differentiate	Jana et al. (2013)
	Film	pMbs *in vitro*	Ability to adhere	-	Iyer et al. (2013)

2.3.2 Polyesters

The most used synthetic polymers in skeletal muscle engineering are poly-α-esters. These polymers contain an aliphatic ester bond in their backbone and are biodegradable by hydrolysis. They have better mechanical properties than natural polymers and are highly processable, meaning that they can be processed as any engineering thermoplastic (melted and formed into fibers, rods and molded parts) (Middleton & Tipton, 2000). The most used and commercially available polyesters include: poly-glycolic acid (PGA), poly-lactide acid (PLA), poly-caprolactone (PCL) and the copolymer poly-lactide-co-glycolic acid (PLGA) (Table 2).

a) PGA

PGA was one of the very first degradable polymers ever investigated for biomedical use. It is a rigid thermoplastic material with high crystallinity (46-50%) (Gunatillake et al., 2003), it has a very high tensile strength (12.5 GPa) and it is often fabricated into a mesh network for tissue engineering applications (Ulery et al., 2011). Saxena et al. demonstrated that mouse primary myoblasts can be seeded onto PGA meshes and be successfully transplanted. These myoblasts were able to survive, organize along the strands of the PGA matrix and regenerate in a period of 6 weeks (Saxena et al., 1999 and 2001). However, some of PGA downsides are: rapid degradation that leads to the rapid loss of mechanical strength and a significant local production of glycolic acid that have been linked to a strong, undesired inflammatory response (Ulery et al., 2011).

b) PLA

PLA possesses chiral molecules, hence it comes in four forms: poly(L-lactic acid) (PLLA), poly(D-lactic acid) (PDLA), poly(D,L-lactic acid) (PDLLA), a racemic mixture of PLLA and PDLA, and meso-poly(lactic acid) (Ulery et al., 2011). PDLA and PLLA are semi-crystalline solids, with similar rates of hydrolytic degradation as PGA (Gunatillake et al., 2003). As far as use in skeletal muscle engineering, only PLLA has been studied. It has a mechanical strength of 4.8 GPa, while the additional methyl group in PLA causes the polymer to be much more

hydrophobic and stable against hydrolysis than PGA, that translates into a slow degradation time (Ulery et al., 2011). Scaffolds of this polymer are often fabricated by electrospinning, producing a mesh of fibers with controlled orientation and diameter. One investigation with electrospun PLLA scaffolds showed that aligned nanoscale topographic features cause the alignment of C2C12 myoblasts and promoted myotube assembly along the nanofibers (Huang et al., 2006). A similar work was done by Cronin et al. (2004) using a PLLA scaffold in human adult skeletal muscle cell line (HSKM5), showing that these cells could adhere and differentiate on the scaffolds. However, one of the main downsides of PLA is its highly acidic degradation products (Ulery et al., 2011).

c) PLGA

PLGA is the most investigated degradable copolymer for biomedical applications because its PLA/PGA composition may be optimized depending on the desired characteristics such as degradation and mechanical strength. For example, the degradation times of 50:50 PLGA, 75:25 PLGA, and 85:15 PLGA are 1–2 months, 4–5 months, and 5–6 months, respectively (Ulery et al., 2011). Similar to PLLA experiments, Aviss et al. (2010) obtained electrospun PLGA scaffolds with aligned orientation. Results showed that these scaffolds provided contact guidance for C2C12 myoblast proliferation, alignment and differentiation.

d) PCL

PCL is a semicrystalline polyester with low tensile strength (23 MPa), but very high elongation at breakage (4700%), making it a very good elastic biomaterial. Because of its very low *in vivo* degradation rate, it has found useful as a long-term implant device (Ulery et al., 2011). Studies with electrospun PCL scaffolds showed that C2C12 cells able to grow proliferate and differentiate on them. Plasma coated scaffolds were identified as strong triggers of differentiation, measured by myotube density, sarcomeric striation and contractility (Guex et al., 2012). Choi et al. (2008) found that unidirectionally oriented PCL/collagen

electrospun nanofibers induced human skeletal muscle cells (hSkMCs) alignment and myotube formation.

Table 2. Polyesters studied for scaffolds in skeletal muscle engineering.

Polymer	Scaffold structure	Cell type and technique	Results		References
			Proliferation conditions	Differentiation conditions	
PGA	3D mesh	Rat primary myoblasts *in vivo*	-	Ability to differentiate along the strands	Saxena et al. (2001); Saxena et al. (1999)
PLLA	Mesh of fibers	C2C12 *in vitro*	Myoblast alignment through fibers. Ability to proliferate	Ability to differentiate. Myotubes oriented as fibers.	Huang et al. (2006)
	Films and mesh	HSKM5 *in vitro*	Ability to adhere	Ability to differentiate	Cronin et al. (2004)
PLGA	Aligned mesh of fibers	C2C12 *in vitro*	Myoblast alignment along the directionality of fibers	Ability to differentiate. More differentiated than positive controls	Aviss et al. (2010)
PCL	Aligned mesh of fibers	C2C12 *in vitro*	Myoblast alignment along the directionality of fibers	Formation of striated myotubes	Guex et al. (2012)

2.3.3 Conducting polymers

Studies on the effect of electric stimulation on proliferation and differentiation of certain cell types had led to the recent study of conducting polymers (CP) in biomedical and tissue engineering applications (Breukers et al., 2010; Guo et al., 2013; Dennis et al., 2001; Qazi et al., 2015). CP are composed by organic monomers that are organized in chains with a conjugated (alternation of single and double bonds) backbone. Both single and double bonds contain a chemically strong, localized r-bond, while double bonds also contain less strongly localized p-bonds. The p-orbitals in the series of p-bonds overlap each other, allowing the electrons to be more easily delocalized and move freely between the atoms, which endow the polymer with metal-like semiconductive properties (Balint et al., 2014). They include polypyrroles (PPy), polythiophenes (PTh) and polyanilines (PANI) (Figure 6), that exhibit high electrical conductivity in the range 10^3 S/cm (Compared to 10^5 for metallic silver and 10^{-8} S/cm for insulators) (Guimard et al., 2007; Wallace & Kane-Maguire, 2002). These polymers are thermally and

chemically stable, and its electric conductivity can be controlled by different doping methods. Doping is the process of oxidizing (p-doping) or reducing (n-doping) a neutral polymer and providing a counter anion or cation (dopant), respectively. Upon doping, a CP system with a net charge of zero is produced due to the close association of the counter ions with the charged CP backbone. This process introduces charge carriers, in the form of charged polarons (radical ions) or bipolarons (dications or dianions), into the polymer (Guimard et al., 2007). As an electrical potential is applied, the dopants start to move in or out of the polymer (depending on the polarity), disrupting the stable backbone and allowing charge to be passed through (Balint et al., 2013). The capacity to reversibly switch between oxidation states, allows control over polymer characteristics including surface energy, conductivity, morphology, and Young's modulus, all of which may be modulated to enhance or control the behavior of responsive cells. Also, the incorporation and release of dopant ions adds an extra dimension of flexibility and functionality to these polymers, in particular, if the dopants are bioactive (Breukers et al., 2010). These conductive properties have allowed PANI, PPy, PTh and its derivatives to be used as scaffolds for electrical stimulation in order to improve proliferation and growth of neurons for neural engineering (Abidian et al., 2012; Guarino et al., 2013). They have also shown good biocompatibility with other cell types, such as fibroblasts (Zhao et al., 2012) and keratinocytes (Humpolicek et al., 2012). Among the disadvantages of CPs are their hidrofobicity and lack of biodegradability (Guo et al., 2013).

For skeletal muscle engineering, electrospun scaffolds of PCL/PANI and poly(L-lactide-co-caprolactone) (PLCL)/PANI have being studied with C1C12 myoblasts, concluding that topographical cues and the electroactivity of nanofibers synergistically stimulate muscle cell differentiation (Jun et al., 2009; Ku et al., 2012). PThs have several interesting and beneficial characteristics such as their ambient stability and the fact that its properties can be modified by the addition of functional groups at the monomer structure (Breukers et al., 2010).

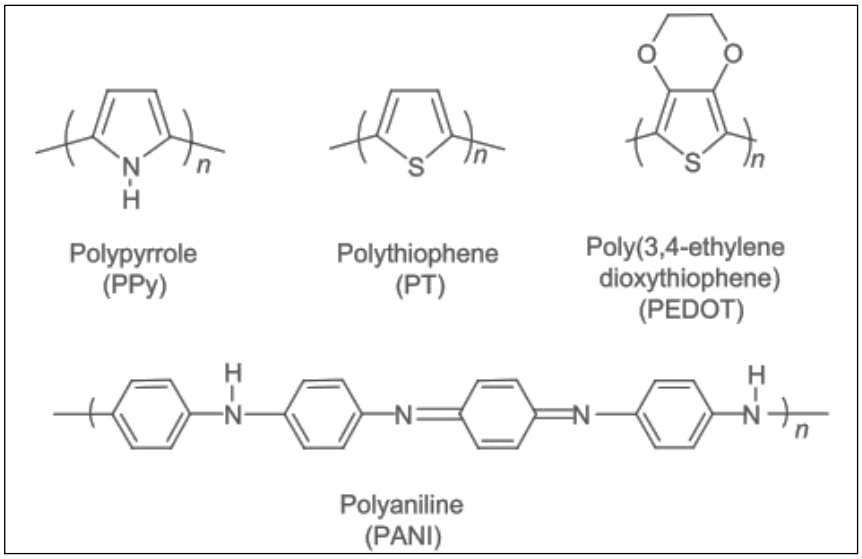

Figure 6. Chemical structures of the most commonly explored CPs for biomedical applications. Obtained from Guimard et al. (2007).

Some researchers tested the use of PThs scaffolds with mice primary myoblasts using three thiophene polymers: Polybithiophene (PBTh), poly-3-decoxythiophene (P3DTh), and poly(decyl 4,4″-di-decoxy-2,2′:5′,2″-terthiophene-3′-carboxylate) (PDTTh3E), finding that they all support primary myoblast proliferation and differentiation *in vitro* (Quigley et al., 2013). Breukers et al. (2010) investigated the effect of films and electrospun fibers of an ester-functionalized organic polythiophene (poly-octanoic acid 2-thiophen-3-yl-ethyl ester (poly(OTE)) and the polymer that results from post-polymerization hydrolysis of the ester linkage (poly 3'-(2-hydroxyethyl) thiophene (poly(HET)). Poly(OTE) and poly(HET) polymer films supported proliferation and differentiation of C2C12 and mouse primary myoblasts (Figure 7) (Breukers et al., 2010). Skeletal muscle engineering studies using CPs are resumed in Table 3.

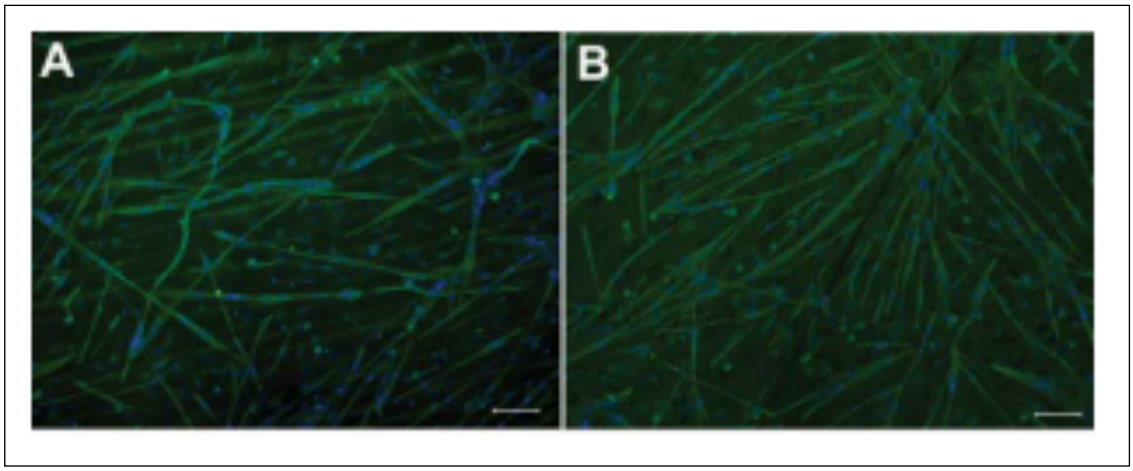

Figure 7. pMbs differentiated on pThs.
A. poly(OTE) or **B.** poly(HET) films doped with pTS. Myofibers were fixed after 3 days in differentiation and stained with an anti-Desmin antibody and the nuclei counterstained with DAPI. Scale bars represent 100nm. Images obtained from Breukers et al. (2010).

Table 3. CPs studied for scaffolds in skeletal muscle engineering.

Polymer(s)	Scaffold structure	Cell type and technique	Results		Reference
			Proliferation conditions	Differentiation conditions	
PLCL/PANI	Mesh of fibers	C2C12 *in vitro*	Ability to proliferate	Ability to differentiate. PANI presence stimulates more differentiation	Jun et al. (2009)
PCL/PANI	Aligned mesh of fibers	C2C12 *in vitro*	Ability to proliferate. Morphology dependent on orientation of fibers	Ability to differentiate. PANI presence stimulates more differentiation	Ku et al. (2012)
PBTh	Film	pMbs *in vitro*	Ability to proliferate as positive controls	Ability to differentiate as controls	Quigley et al. (2013)
P3DTh	Film	pMbs *in vitro*	Ability to proliferate as positive controls	Ability to differentiate as controls	Quigley et al. (2013)
PDTTh3E	Film	pMbs *in vitro*	Ability to proliferate as positive controls	Ability to differentiate as positive controls	Quigley et al. (2013)
Poly(OTE)	Films and aligned mesh of fibers	C2C12 and pMbs *in vitro*	Both cell types adhere and proliferate as well than controls on films.	Primary myoblasts were able to differentiate on polymer films and mesh of fibers	Breukers et al. (2010)
Poly(HET)	Films and aligned mesh of fibers	C2C12 and pMbs *in vitro*	Both cell types adhere and proliferate as well or better than controls on films and mesh of fibers	Primary myoblasts were able to differentiate on polymer films and mesh of fibers	Breukers et al. (2010)

2.4 Motivation

After an extensive review of all the different polymers used in skeletal muscle engineering, including their advantages and disadvantages, chitosan captured our special attention due to its widely known beneficial properties, as described above, and also due to the fact that it is a low cost material and can be obtained by unlimited resources, mainly crustacean shell waste (Kaur & Dhillon, 2013). Preliminary studies were assessed, in order to evaluate the biocompatibility of medium molecular weight chitosan films with C2C12 and mouse primary myoblasts (pMbs). We found that chitosan showed poor myoblast adhesion, compared to positive controls, especially compared with culture in polystyrene (PS) tissue culture plates, and also differentiation was not observed (Figure 8 and 9). On the other hand, we found that PLGA 75:25 films (used as internal positive controls) showed an extremely different myoblast adhesion when comparing C2C12 and pMbs (Figure 9 and 10). These studies showed two important issues to assess. The first one was the low adhesion of chitosan with myoblasts, and the second was the big differences found between C2C12 and primary myoblasts response with 2 different polymer films.

The use of polymer blends has attracted attention for producing polymer scaffolds in tissue engineering because they can exhibit a combination of characteristics related to each polymer employed. In order to improve myoblast adhesion and biocompatibility on chitosan films, we propose to use chitosan blended with the CP poly(OTE). This polymer was chosen for its ease of synthesis, solubility in common organic solvents, its inherent electroactive properties and previous reports that showed good biocompatibility with C2C12 and pMbs (Breukers et al., 2010). As discussed before, C2C12 is an immortalized myoblast cell line that is commonly used in cell biology as a simplified model to study myogenic progression *in vitro*, however due to its modifications the biology of the cell is altered, affecting gene and protein expression. Due to this matter, it is important in this work to consider and evaluate biocompatibility with both mouse myoblast cell types to determine how differently they behave in the polymer films and to determine which cell model is more appropriate for human applications if they differ greatly.

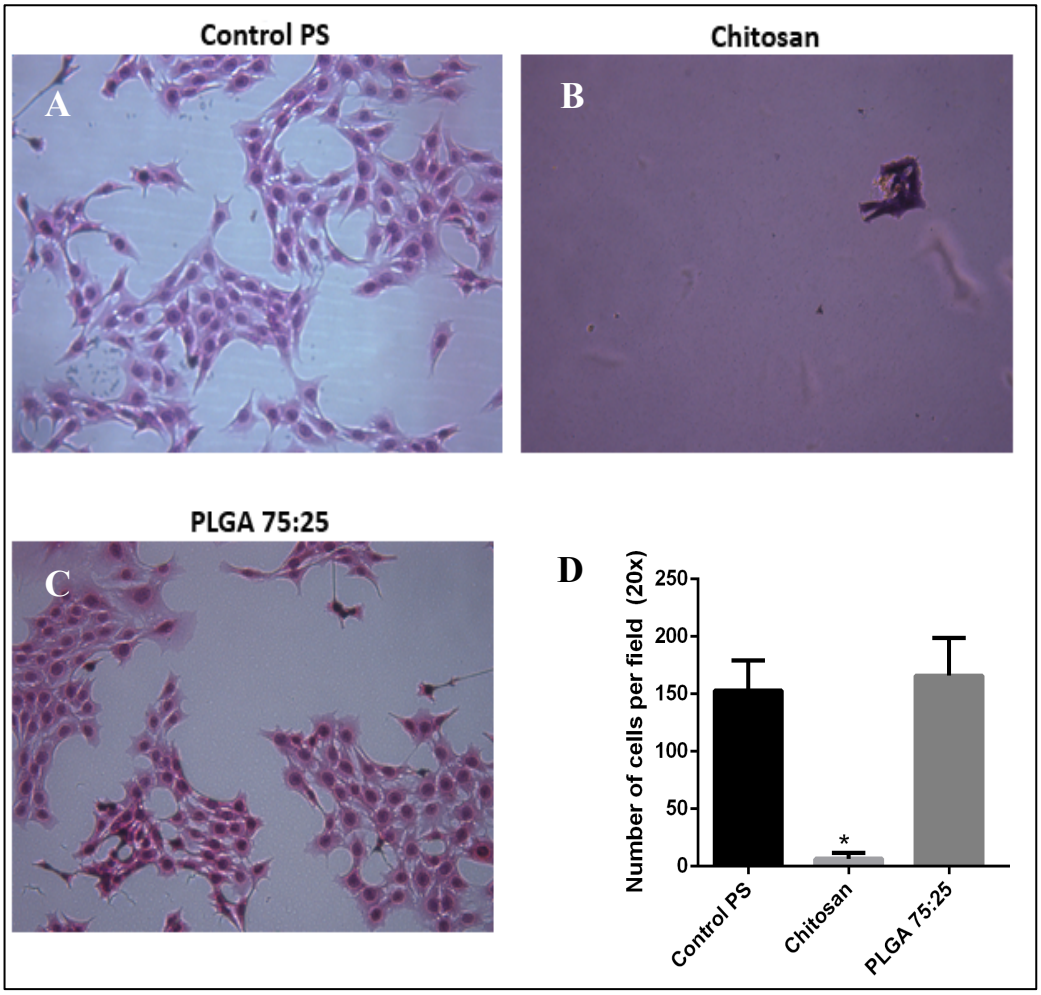

Figure 8. C2C12 myoblast culture on chitosan and PLGA 75:25 films in proliferative conditions. Cells were cultured on proliferative conditions for 2 days (in duplicate) for each polymer film and then fixed and stained with hematoxylin-eosin. **A.** 20X representative image of C2C12 on polystyrene (PS) as positive control. **B.** 20X representative image of C2C12 on a chitosan films. **C.** 20X representative image of C2C12 on PLGA 75:25 films. **D.** Images quantification using Image J software.

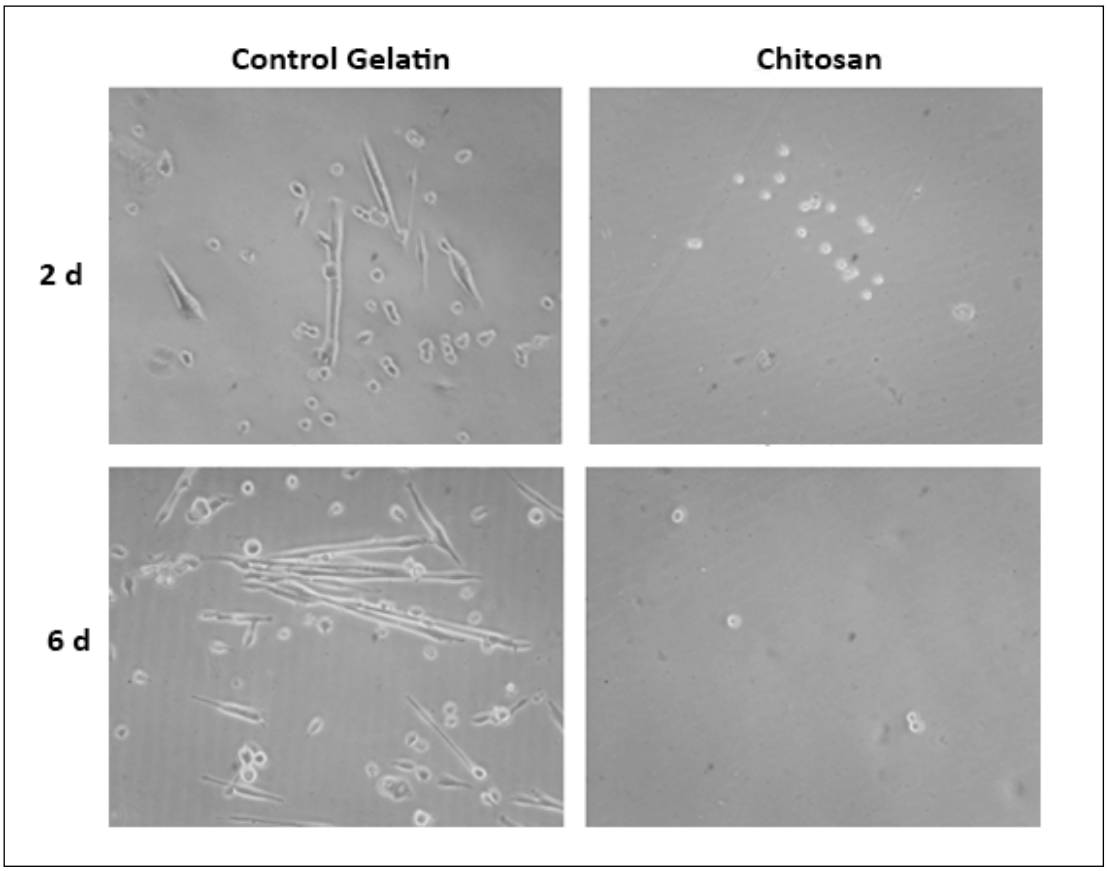

Figure 9. pMbs cultured on chitosan films.
20X images of mouse primary myoblasts cultured for 1 and 6 days on chitosan films. Gelatin coated cell culture plates were used as positive control.

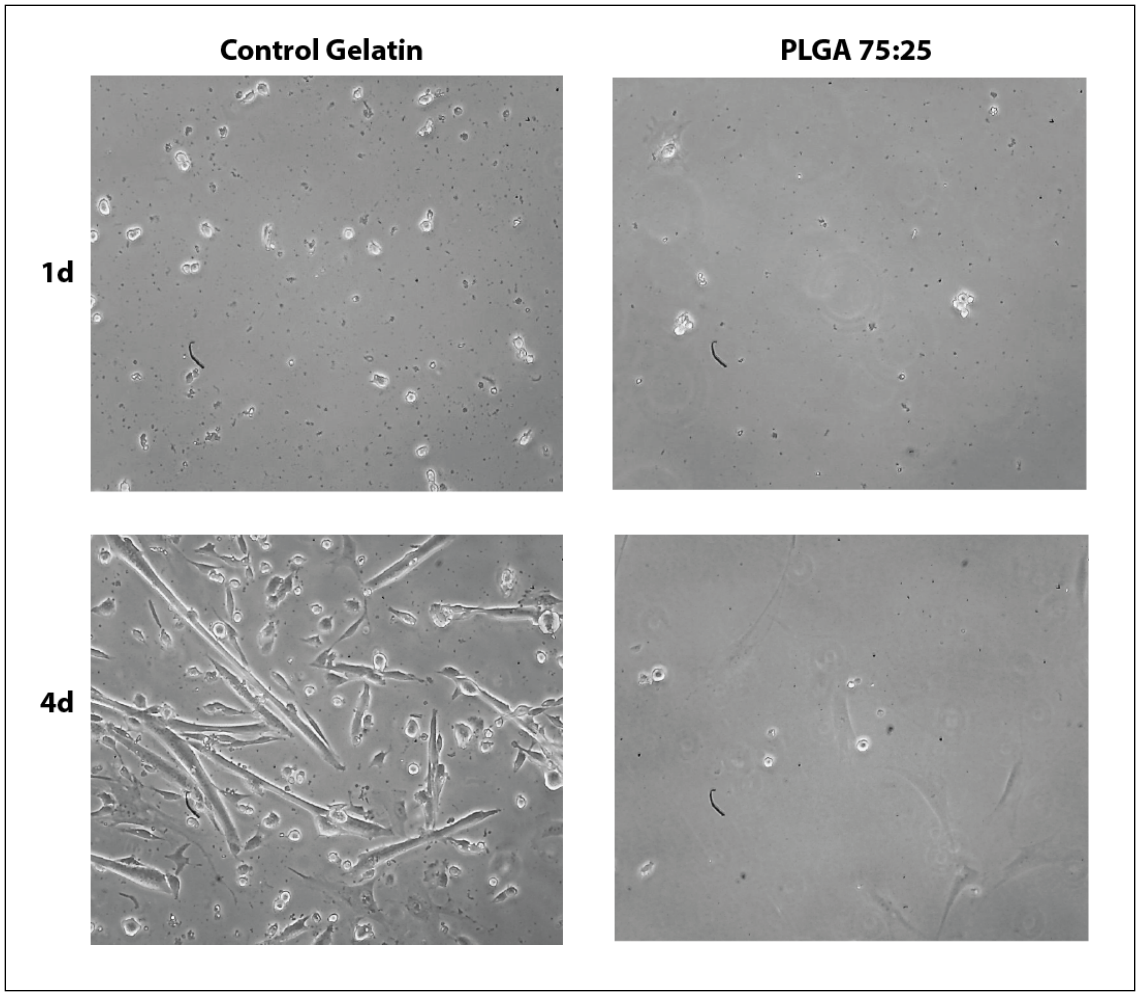

Figure 10. pMbs cultured on PLGA 75:25 films.
20X images of mouse primary myoblasts cultured for 1 and 4 days on PLGA 75:25 films. Gelatin coated cell culture plates were used as positive control.

3 HIPOTHESIS

Considering that chitosan and poly(OTE) are both biocompatible and the combination of both polymers could present the sum of their beneficial properties, we propose that poly(OTE) blended with chitosan improves murine myoblast adhesion and survival *in vitro*.

4 OBJECTIVES

4.1 General objective

The main objective of this work is to obtain and characterize films of chitosan and chitosan/poly(OTE) blends to determine their cell response with skeletal muscle progenitors *in vitro*, directly comparing the adhesion and survival of the C2C12 cell line and adult mouse primary myoblasts.

4.2 Specific objectives

I. Chitosan and chitosan/poly(OTE) film fabrication and chemical characterization by Fourier Transform Infrared Analysis (FT-IR).

II. Determination of films surface morphology by Atomic Force Microscopy (AFM) and Scanning Electron Microscopy (SEM).

III. Evaluation of biological response of both cell types on chitosan/poly(OTE) films:

- Determination of protein adsorption using bicinchoninic acid assay (BCA).
- Determination of myoblast adhesion and survival on polymer films on proliferation and differentiation conditions.
- Evaluation of lineage specific protein expression of cells cultured on polymer films, using western blot and immunofluorescence.

5 METHODS

5.1 Poly(OTE) synthesis

OTE monomer was synthetized as described in Camurlu et al. (2005). Stoichiometric amounts of 3-thiophene-ethanol and triethylamine were dissolved in dichloromethane. Octanoyl-chloride was added dropwise to the mixture and the reaction was kept with stirring for 6 hours (Figure 11). After purification, OTE was characterized by ^{1}H and ^{13}C Nuclear magnetic resonance (NMR) spectroscopy and Infrared (IR) spectroscopy.

Poly(OTE) was synthetized with a modified methodology based on Camurlu et al. (2005) and Breukers et al. (2010). OTE was dissolved in chloroform and $FeCl_3$, used as oxidizing reagent, in nitromethane was added dropwise at 0ºC under nitrogen atmosphere. This temperature was maintained during the first 3 hours of the reaction, and the system was maintained in agitation for 24 hours (Figure 11). The polymer was precipitated in methanol, separated by decantation and washed several times to remove oxidant and monomer remains. Poly(OTE) was characterized by IR spectroscopy before using in cell culture.

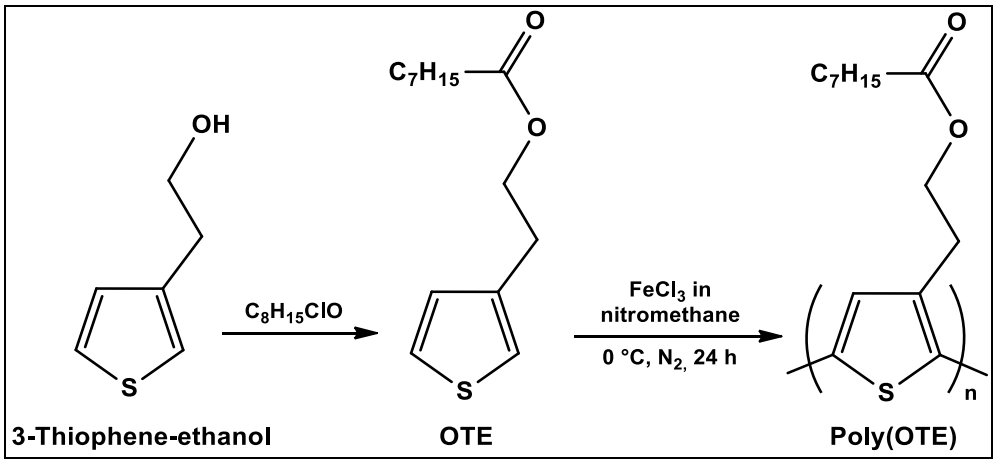

Figure 11. Summary of OTE and poly(OTE) synthesis.
Modified from Breukers et al. (2010).

5.2 Polymer film preparation

Poly(OTE) and chitosan films were casted and used for comparison purposes. Chitosan films were prepared in tissue culture plates by solvent casting, dissolving chitosan (medium molecular weight, 75-85% deacetylated, Sigma-Aldrich) in 0.5 M acetic acid. Each film of 10 μm thickness, contained 2.5 mg chitosan/cm^2, as described in Lieder et al., (2013). Overnight dried chitosan membranes were neutralized by incubating with 0.5M NaOH for 30 minutes, followed by 3 washes with double distilled water. Chitosan films preparation was standardized using Alizarin red stain as quality control (Lieder et al., 2013).

Poly(OTE) films were prepared by solvent casting poly(OTE) 0.25%w/v solution in chloroform to form films containing 0.02mg poly(OTE)/cm^2. For chitosan/poly(OTE) blends, poly(OTE) was dissolved in chloroform for approximately one hour and then mixed with a chitosan 1%w/v in 0.5M acetic acid solution and stirred overnight. Once the emulsion was homogenized, chitosan/poly(OTE) blends were casted and neutralized as described for chitosan.

For cell culture, polymer films were sterilized 30 minutes with 70% ethanol and 30 minutes with UV-light. Prior to cell seeding, films were equilibrated with proliferation medium (see myoblast cell culture section) for 20 min. Uncoated polystyrene (PS) or gelatin coated PS wells were used as positive controls of cell adhesion for C2C12 and primary myoblasts, respectively. For chemical and structure analysis, polymer films were casted in 18mm diameter glass coverslips, treated and sterilized as described for cell culture plates.

5.3 Fourier Transform Infrared Analysis (FT-IR)

FT-IR spectra of polymer films were obtained using a Vector 22, Bruker spectrometer. The spectra were obtained in the range 400 a 4000 cm^{-1}. OPUS software was used to control the spectrometer and spectrum analysis.

5.4 Scanning Electron Microscopy (SEM)

To analyze films superficial morphology, polymer films casted on coverslips were sputter coated with gold and then analyzed by SEM (FEI Inspect F50) using 4000X magnification.

5.5 Atomic Force Microscopy (AFM)

Two dimension (2D) and three dimension (3D) images, as well as roughness data were obtained using an AFM microscope (Bruker Innova). Images of 10 x 10 µm areas were obtained in tapping mode using a silicon tip without covering (Asylum Research AC240TS-R3). Mean film height (R_a) was calculated (from 3-5 areas per polymer film) as follows:

$$R_a = \frac{1}{N}\sum_{i=1}^{N}|Z_i - Z| \qquad (1)$$

where Z_i was the height of a given point of the area analyzed, Z was the average height of the area, and N are the numbers of points analyzed.

5.6 Protein adsorption assay

Polymer films casted in 12 well cell culture plates were incubated with DMEM 10% FBS for 24 hours at 37ºC with 5% CO_2. After the incubation, they were washed two times with phosphate buffered saline (PBS) solution and surface adsorbed proteins were eluted with 500µL of 2% SDS for 4 hours. Before protein quantification, each sample was centrifuged at 15,200 rpm for 20 minutes. The protein concentration in the eluate was measured by BCA.

5.7 Myoblast culture

C2C12 (ATCC CRL-1772) cell line was maintained in DMEM (Life technologies) with 10% fetal bovine serum (FBS) (proliferation medium) (Hyclone) at 37ºC with 5% CO_2. For proliferation assays C2C12 myoblasts were plated at 1500-2500 cells/cm^2 and cultured for 2-3 days. For differentiation assays C2C12 myoblasts were cultured for 3 days in proliferation medium prior to differentiation induction with DMEM 5% horse serum (HS) (Hyclone).

Adult mouse primary myoblasts (pMbs) were obtained as previously described (Olguin & Olwin, 2004) and cultured in F12-C (Life technologies) supplemented with 15% HS and 1nM FGF-2 (proliferation medium) and maintained at 37ºC with 5% CO_2 and 6% O_2. For differentiation assays, myoblasts were cultured in growth medium without FGF-2. This procedure was approved by the ethics committee of the University's Biological Sciences Department.

Human primary myoblasts (hMbs) were obtained from healthy patients between 18-60 years old submitted to Pontificia Universidad Católica de Chile's Clinical Hospital for anterior cruciate ligament reconstruction surgery. Only patients that were willing to donate muscle tissue extracted during the procedure were included. Detailed conditions, purpose and utilization of the muscle sample were detailed on an informed consent (Annex F). The biopsy obtained was kept on sterile saline solution at 4ºC until processed. The procedure of grinding and enzymatic treatment for obtaining hMbs was similar to pMbs. hMbs were cultured in F10 (Life technologies) supplemented with 20% SBF and maintained at 37ºC with 5% CO_2 and 6% O_2. For differentiation assays, hMbs were cultured in DMEM supplemented with 4% SFB. This procedure was approved by the ethics committee of the University's Medical School.

5.8 Cell quantification

Cells were plated on polymer films and cultured for 2-3 days in proliferation conditions. They were fixed with paraformaldehyde (PFA) 4% in PBS for 20 min at room temperature, permeated with triton X-100 0.2% in PBS for 10 min. C2C12 myoblasts were stained with Hematoxylin-eosin (H&E) and pMbs were stained with methylene blue. Each sample was divided in 5 quadrants and 2 fields were photographed from each quadrant using Olympus CKX41SF microscope with a MEM 1300 camera (20X magnification). Cells were counted using Image J and the average of each field and quadrants were used to calculate cell number in each sample.

5.9 Cell rescue assay

2500 cells/cm^2 were plated into polymer films and were cultured for 2 days until 60% of confluence was reached. Cells were detached from films with trypsin-EDTA 2X,

counted with trypan blue exclusion method and seeded in cell culture plates without polymer films. After 2 days, differentiation was induced and cultured for 5 days until fixation or protein extraction.

5.10 Western Blot

Cells were lysed in modified radioimmunoprecipitation assay buffer (RIPA) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% IGEPAL, protease, and phosphatase inhibitors). 10–20 μg of total protein was loaded into 10% SDS-Polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes for Western blotting with the following primary antibodies and dilutions: mouse monoclonal anti-Pax7 1:5, mouse monoclonal anti-MHC (MF20) 1:10, mouse monoclonal anti-myogenin (F5D) 1:10 (Developmental Studies Hybridoma Bank), mouse monoclonal anti-GAPDH (EMD Millipore), mouse monoclonal anti-tubulin, 1:10,000 (Sigma-Aldrich) and rabbit polyclonal anti-MyoD (C-20) (Santa Cruz Biotechnology). Secondary antibodies used were: HRP-conjugated anti-mouse IgG and anti-rabbit IgG (Cell Signaling) 1:5,000. HRP activity was detected using the SuperSignal WestDura Extended Duration Substrate (Thermo Fisher Scientific).

5.11 Immunofluorescence

Cells were fixed in PFA 4% for 20 minutes and subjected to standard indirect immunofluorescence (Olguín et al., 2007). Mouse monoclonal anti-MHC (MF20) 1:2 was used as primary antibody and goat anti-mouse Alexa488, 1:500 (Life technologies) as a secondary antibody. Lastly, Vecta-shield with DAPI (Vector labs) was used for mounting. Images were acquired using an epifluorescence microscope Motic® BA410.

6 RESULTS

6.1 Film fabrication and chemical characterization

For the fabrication of chitosan/poly(OTE) blends, 3 and 6% w/v poly(OTE) in chitosan solutions were chosen, since at bigger concentrations no big differences in cell adhesion were observed (data not shown), also it was difficult to obtain stable emulsions at higher concentrations.

To determine whether chitosan and poly(OTE) were interacting on the blends, FT-IR spectroscopy analysis was assessed. Chitosan films spectrum showed a characteristic amino group band at 1651 cm^{-1} and a wide band at 3100-3500 cm^{-1}, corresponding to N-H and O-H bonds, respectively, and another band at 2879 cm^{-1} attributed to the C-H stretching, consistent with the literature. Signals between 898 and 1150 cm^{-1} were observed due to the saccharine structure in the macromolecule (Aghabarari et al., 2013; Zakaria et al., 2012) (Figure 12). Chitosan/poly(OTE) blends spectra showed predominant signals from chitosan, which was expected due to the large chitosan content in the blends. The presence of poly(OTE) was evidenced by a carbonyl signal, corresponding to the ester group present in OTE structure, at approximately 1735 cm^{-1}. The higher the poly(OTE) proportion the higher the signal intensity (Figure 12).

FT-IR spectra of polymer blends showed only signals from the two separated polymers without displacement or new signals. This implies that the two polymers are not interacting in the films.

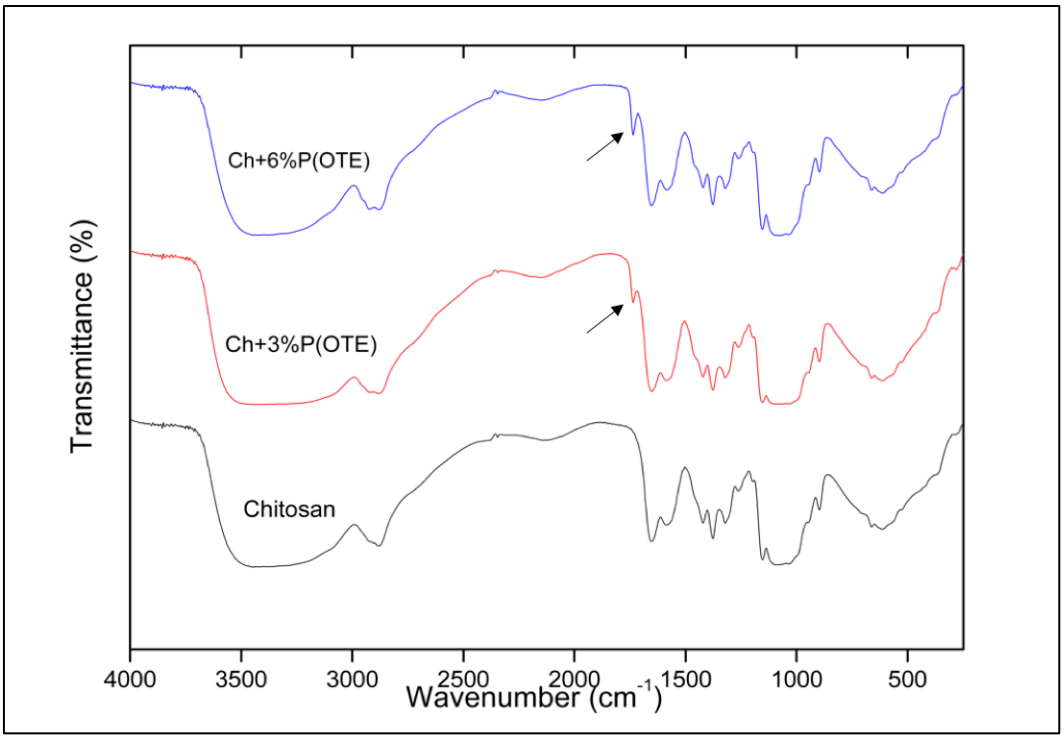

Figure 12. FT-IR spectra of chitosan and chitosan/poly(OTE) films.
Arrows indicate the carbonyl group (C=O) peak from poly(OTE) structure. Poly(OTE) film spectrum available on Annexes (Annex A).

6.2 Determination of films surface morphology

SEM images confirmed that chitosan and poly(OTE) did not interact with each other on the blends, showing domains of poly(OTE) dispersed in chitosan. These had different diameters that depended on poly(OTE) concentration on the blend (Figure 14A and B). Poly(OTE) domains on the 3% blend had a maximum diameter of 2.70 ± 0.21 µm and an average of 1.78 ± 0.66 µm. Domains on 6% blends were bigger and less uniform in size, with an maximum diameter of 8.35 ± 1.18 µm and an average of 3.02 ± 2.53 µm.

Film surface topography was analyzed by AFM and the roughness parameter R_a was obtained for each polymer film. Chitosan films were homogenous and poly(OTE) films showed an uneven distribution, with a tendency to form aggregations, consistent with AFM images shown on Breukers et al. (2010). Blends on the other hand, showed poly(OTE) as the microcomponent represented by higher domains, which were larger in diameter and height on the 6% poly(OTE) blend (Figure 15). Roughness analysis

showed that chitosan and poly(OTE) films were smoother than the blends, with R_a values of 9.15 ± 8.41 and 14.18±1.87 nm, respectively. The presence of poly(OTE) on chitosan blends provoked an increase on roughness, which is higher with higher poly(OTE) proportion on the blend, with R_a values of 15.03 ± 2.91 nm for chitosan/3% poly(OTE) and 28.27 ± 11.99 nm for chitosan/6% poly(OTE).

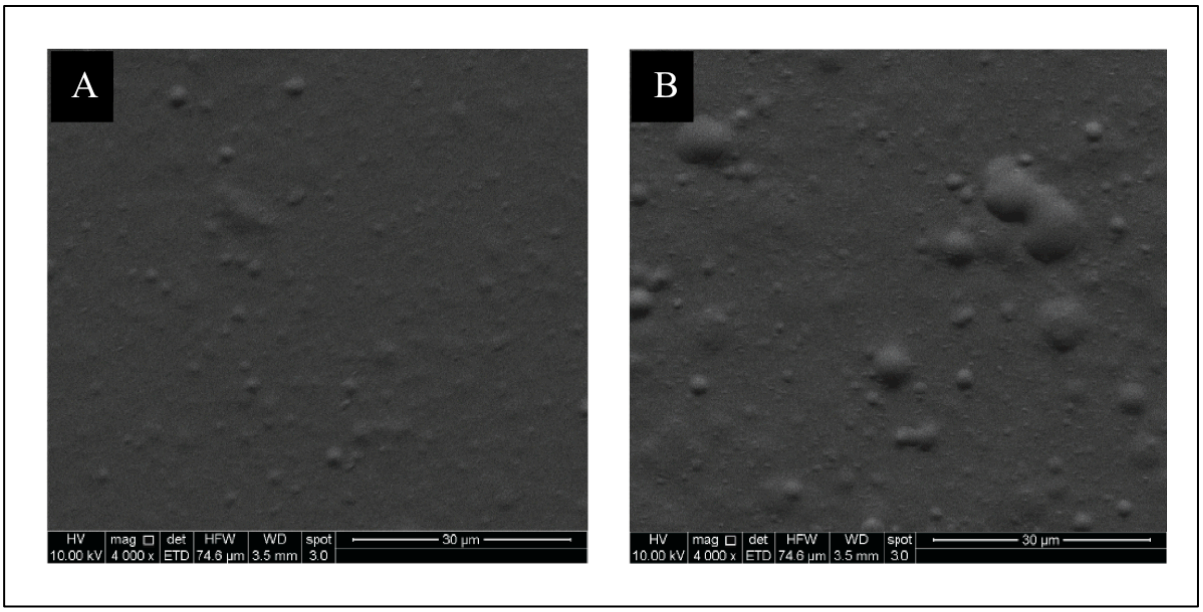

Figure 13. SEM images of chitosan/poly(OTE) films.
A. Chitosan / 3%Poly(OTE) blend, **B.** Chitosan / 6%Poly(OTE) blend.

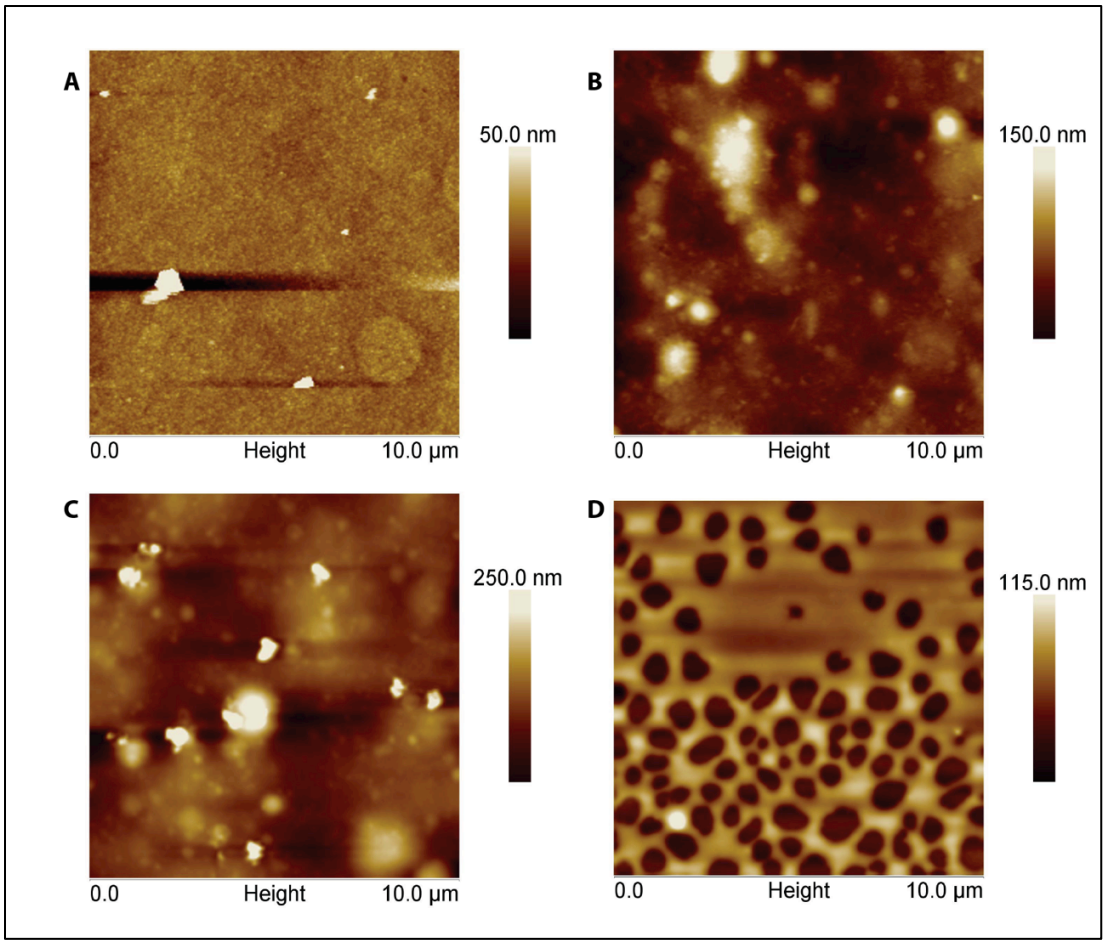

Figure 14. AFM 2D topographic images of all polymer films.
A. Chitosan, **B.** Chitosan/3%Poly(OTE) blend, **C.** Chitosan/6% Poly(OTE) blend and **D.** Poly(OTE). Polymer films were scanned over 10 X 10 µm areas. 3D images available on annexes (Annex B-E).

6.3 Evaluation of polymer films biological response

6.3.1 Determination of protein adsorption on polymer films

Since protein adsorption on polymers surface is the first step on the biocompatibility response, a protein adsorption assay was assessed. Using the BCA it was determined that no significant differences (p-value= 0.23) were observed on polymer films cultured for 24hrs on DMEM 10% FBS (Figure 15).

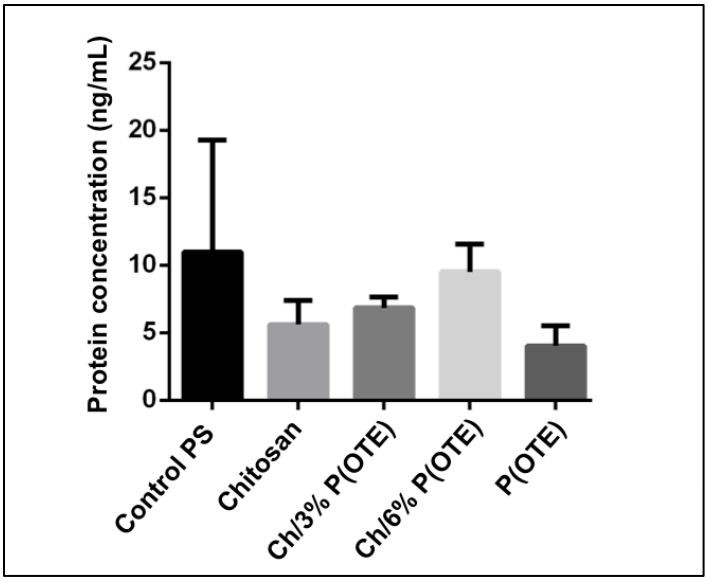

Figure 15. Protein adsorption assay on polymer films.
Protein concentration was determined using BCA method. Error bars represent standard deviation counts from three measures. Each polymer film was analyzed by duplicate. Ch=chitosan; P(OTE)=poly(OTE).

6.3.2 C2C12 culture on proliferative and differentiation conditions

3% and 6% poly(OTE) blended with chitosan suggested an increase on C2C12 adhesion. Even though cell adhesion was improved by these blends, poly(OTE) still presented higher C2C12 adhesion. Cell morphology was also affected by the presence of chitosan and chitosan/poly(OTE) blends, observing clustered cells with an elongated morphology (Figure 16A). Western blot analysis showed that cells cultured on chitosan/poly(OTE) films had an increase in Pax7 expression, while MyoD levels were similar, with the exception of chitosan films (Figure 16B).

Differentiation was not observed on chitosan or chitosan/poly(OTE) films, with an increase on cell clustering and an exacerbated elongated morphology for chitosan/poly(OTE) films. Besides, some cells were detached from chitosan films, and because cells exhibit low adhesion, practically no cells were observed under these conditions (Figure 17A). Western blot analysis showed that Pax7 levels were still higher than PS, but MyoD was also higher on the polymer films. Myogenin was expressed in all polymer films and PS, but MHC was not expressed on cells cultured on the blends (Figure 17B).

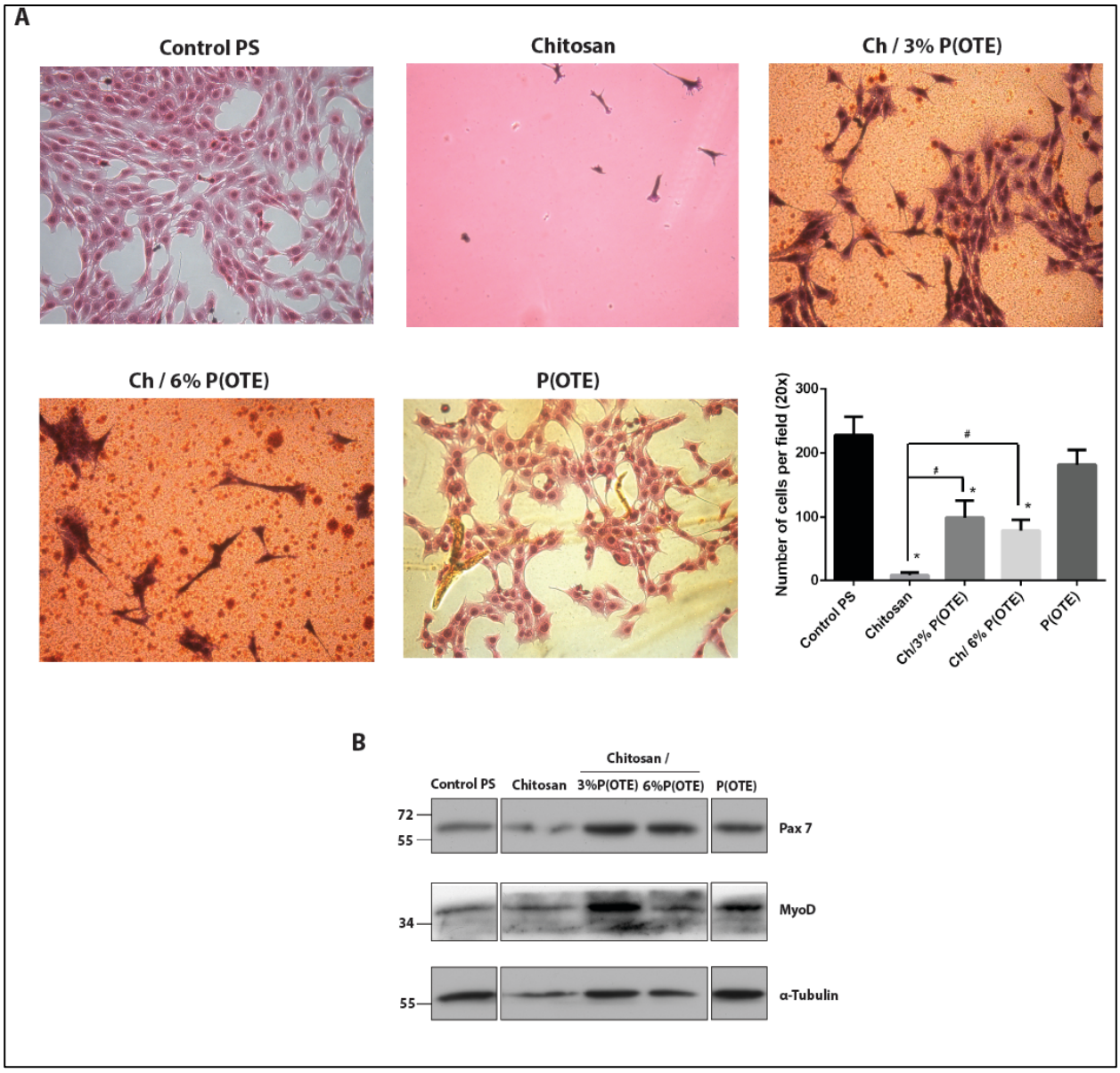

Figure 16. C2C12 culture on all polymer films in proliferative conditions**.**
Cells were cultured on proliferation media for 3 days (in duplicate) for each polymer film and then fixed and stained with hematoxylin-eosin. **A.** 20X representative images of C2C12 cultured on each polymer film and image quantification. Error bars represent standard deviation counts from ten images for each duplicate sample. N=2. One-way ANOVA, * denotes significant difference ($P < 0.05$) with control PS; # denotes significant difference ($P < 0.05$) with chitosan. **B.** Western blot of Pax7 and MyoD expression. α-tubulin was used as a loading control. Ch=chitosan; P(OTE)= poly(OTE).

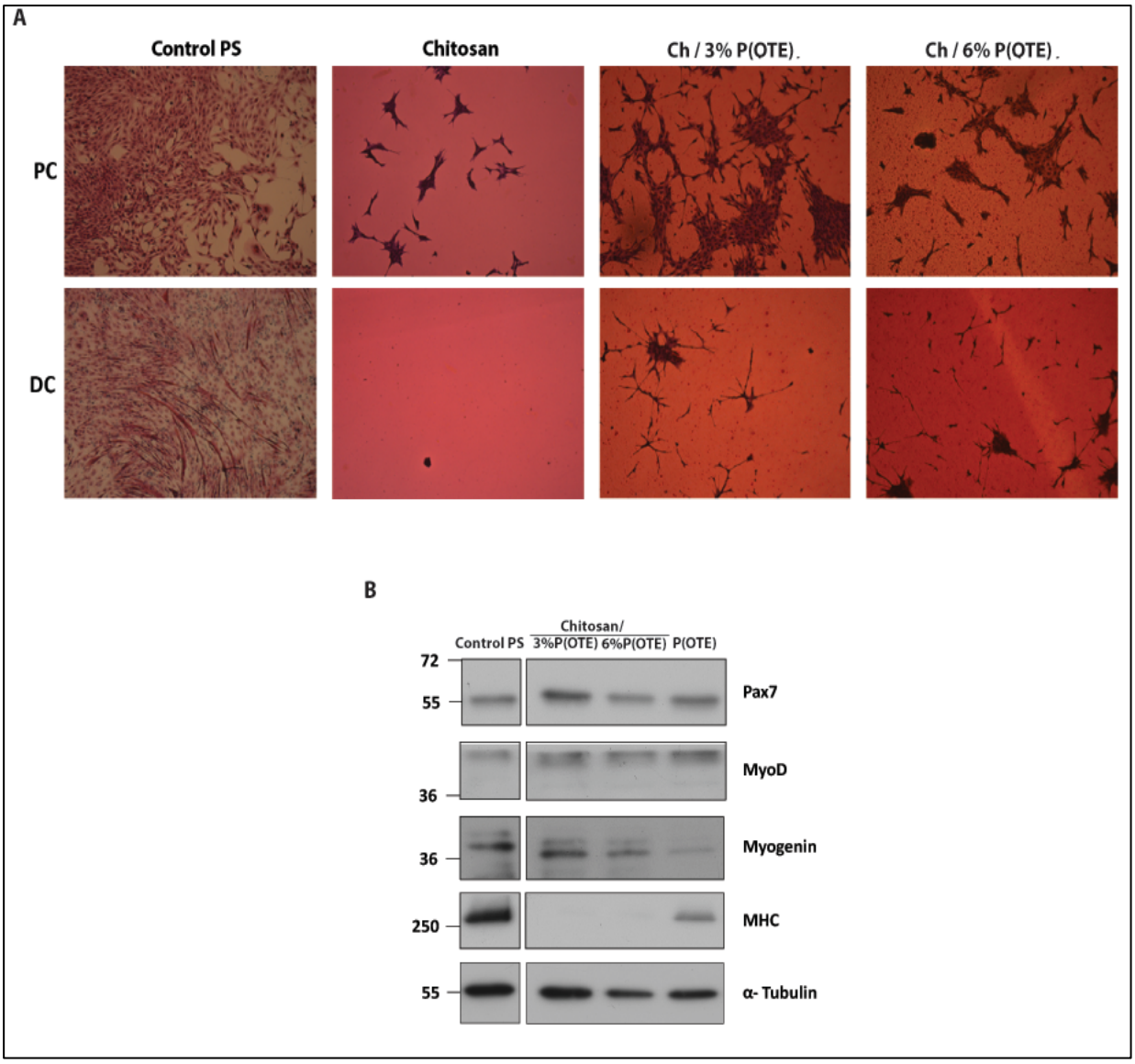

Figure 17. C2C12 myoblasts culture on all polymer films in differentiation conditions.
Cells were cultured on proliferation media for 3 days (in duplicate) for each polymer film and then fixed and stained with hematoxylin-eosin. Simultaneously, other samples were cultured on proliferation media for 3 days (also in duplicate) and then subjected to culture on differentiating conditions for 3 more days before fixation and stain. N=2. **A.** 20X representative images of C2C12 cultured on each polymer film. PC= proliferative conditions. DC=differentiating conditions. **B.** Western Blot of Pax7, myoD, myogenin and MHC expression of 5 day differentiating C2C12 cells cultured on different polymer films. α-tubulin was used as a loading control. Ch=chitosan; P(OTE)= poly(OTE).

To determine if these cells maintain their differentiation potential, a cell rescue assay was performed. After 5 days on differentiation media, it was observed that cells grown on all polymer films differentiate into myotubes (Figure 18A). This experiment showed that C2C12 myoblasts maintain their ability to differentiate after cultured on chitosan and chitosan/poly(OTE) films. Protein expression showed a similar level of Pax7 on all polymer films cultures, which was lower than the PS control. All cells expressed differentiation markers as myogenin and MHC (Figure 18B).

6.3.3 pMbs culture on proliferative and differentiation conditions

Results showed a higher number of adhered primary myoblasts on chitosan than C2C12 did (Figure 16A and 19A). Only in 3% poly(OTE) blended with chitosan, pMbs cell number was significantly improved when compared with chitosan. (Figure 19A). There was practically no pMbs adhesion on poly(OTE) films compared with C2C12 cultures. Western blot analysis showed a similar trend between pMbs and C2C12 cultures for chitosan and 3% poly(OTE) films on Pax7 levels (Figure 19B). MyoD levels were not detected probably because of the low cell density on this culture conditions.

After 2 days of culture differentiation was not observed (Figure 20). Cells were clustered on blends and detached on the case of chitosan, as seen for C2C12.

Cell rescue assay showed that these cells grown on all polymers maintained their ability to differentiate (Figure 21A). Protein expression showed similar levels of Pax7, on chitosan and 3% blend, and higher levels on gelatin treated plates, equivalent to observations obtained on C2C12 cultures. Myogenin and MHC were expressed on all conditions except for poly(OTE), due to low confluence (Figure 21B).

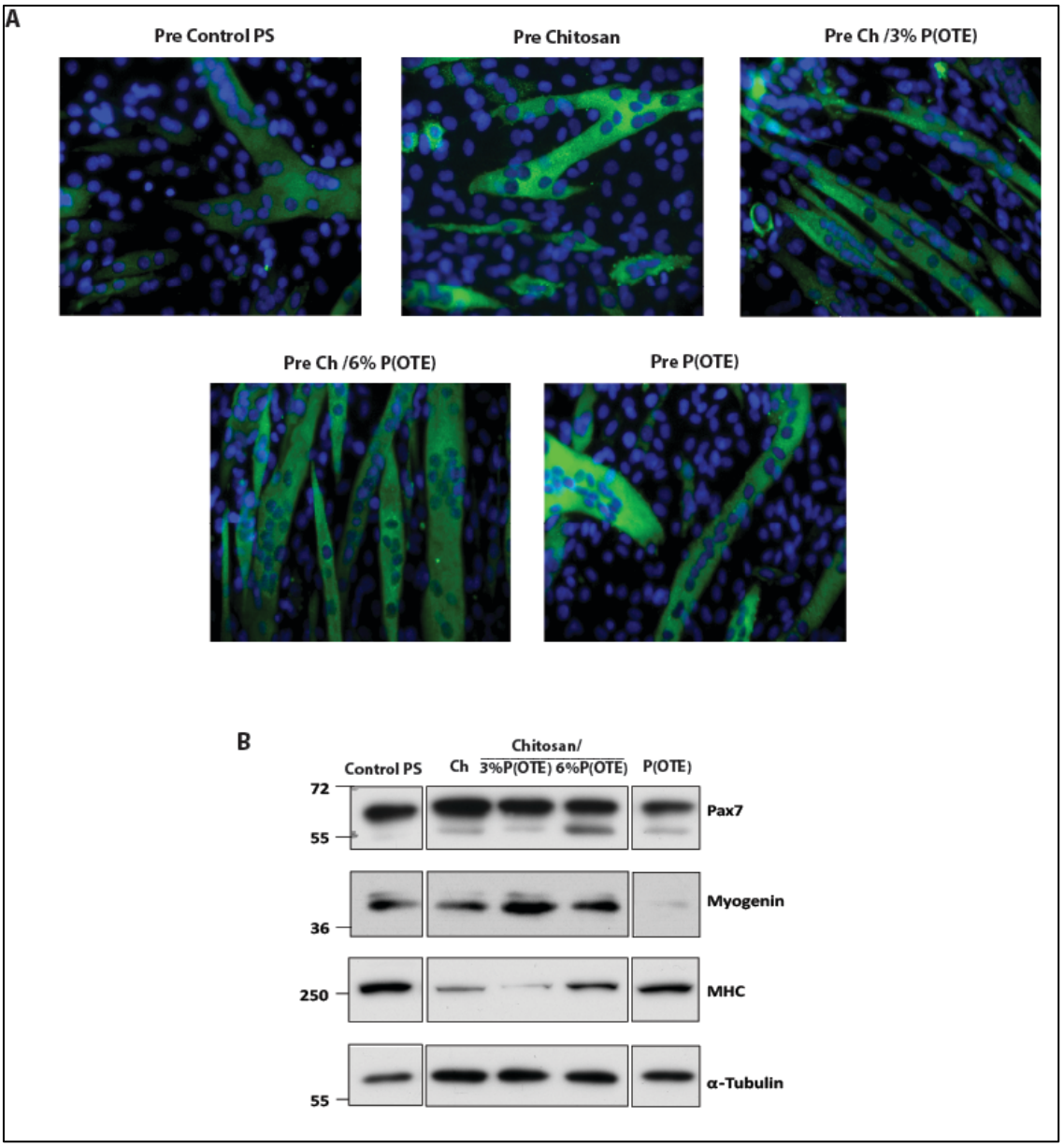

Figure 18. Determination of C2C12 differentiation potential *in vitro*.
A. 40X representative images of MHC expression detected by immunofluorescence. Myoblasts were cultured on polystyrene culture plates for 5 days on differentiation media after their culture on different polymer films for 2 days. N=2. Myotubes were fixed and immunostained with an anti-MHC antibody. Nuclei were counterstained with DAPI. **B.** Western Blot of Pax7, myogenin and MHC expression after 5 days on differentiation media. α-tubulin was used as a loading control. PRE= Pre cultured on; Ch=chitosan; P(OTE)= poly(OTE).

6.3.4 Culture of human primary myoblasts on polymer blends

To determine which cell type would be a better cell model for human application, preliminary experiments using hMbs were assessed. Results showed that hMbs adhered and proliferate similar to C2C12 on all studied polymer films, however the cell number on poly(OTE) films was significantly lower than the control (Figure 28). hMbs presented elongated morphologies on chitosan/poly(OTE) blends, but specially on chitosan.

Results showed that after 4 days of culture, differentiation was not observed on chitosan or chitosan/poly(OTE) films. There was a cell cluster formation on chitosan/poly(OTE) blends and an exacerbation on the morphology seen in proliferation conditions, as observed in C2C12 myoblasts (Figure 29).

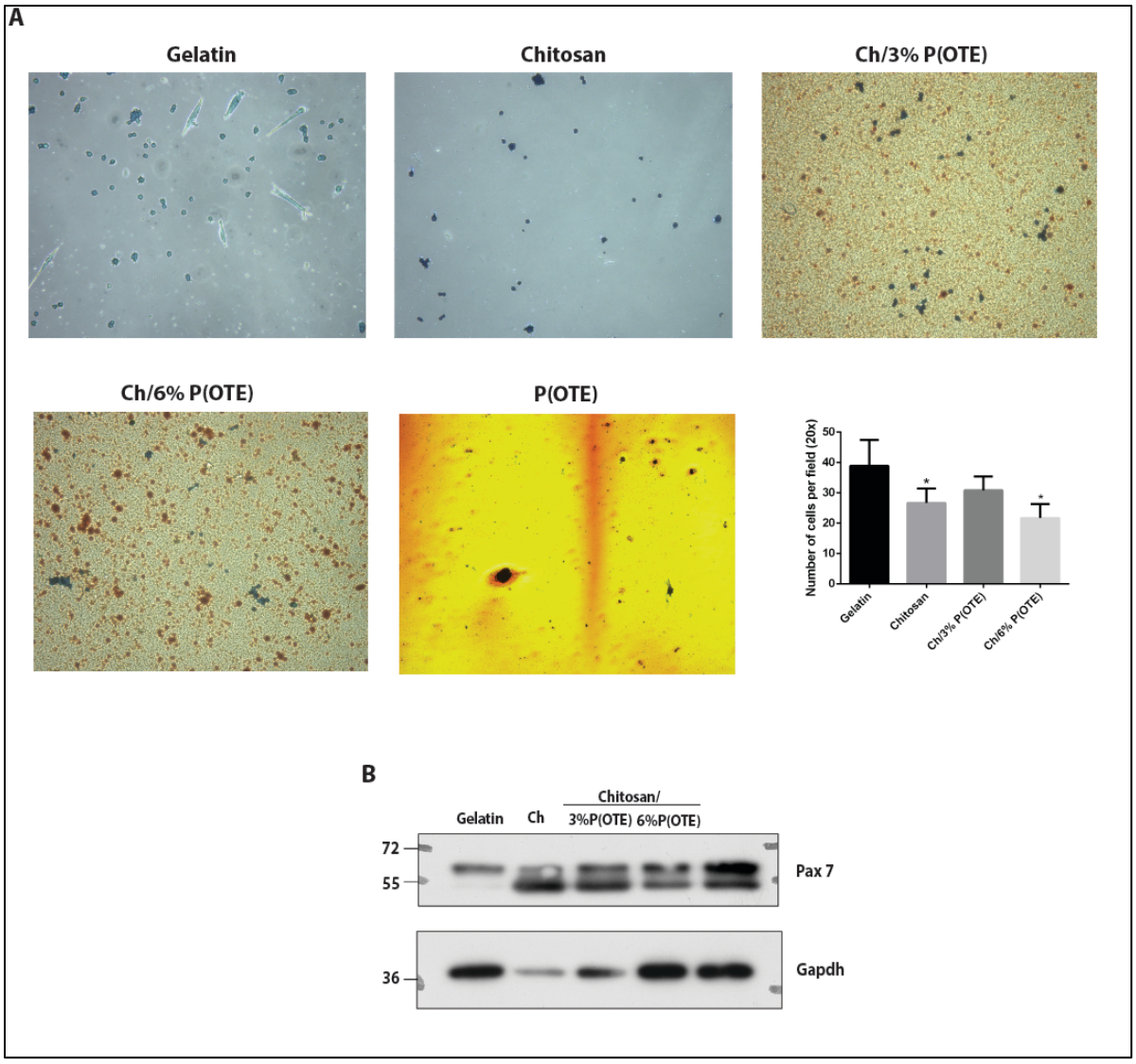

Figure 19. pMbs culture on all polymer films in proliferative conditions.
Cells were cultured on proliferation media for 2 days (in duplicate) for each polymer film and then fixed and stained with methylene blue. **A.** 20X representative images of pMbs cultured on each polymer film and image quantification. Error bars represent standard deviation from ten images for each duplicate sample. N=2**.** One-way ANOVA, *denotes significant difference ($P < 0.05$) with control gelatin. **B.** Western blot of Pax7 expression. Gapdh was used as a loading control. Ch=chitosan; P(OTE)= poly(OTE).

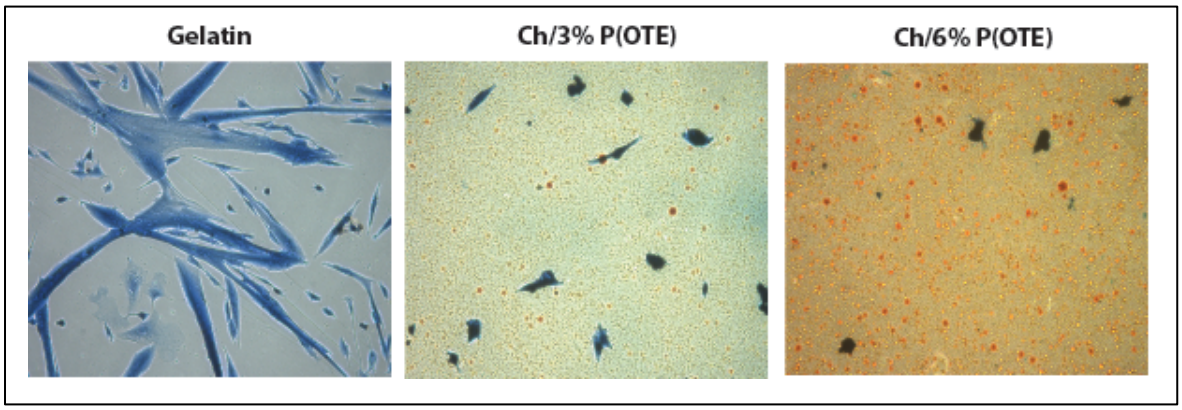

Figure 20. pMbs culture on polymer films in differentiation conditions.
20X representative images of C2C12 cultured on each polymer film. Cells were cultured on proliferation media for 2 days (in duplicate) for each polymer film and then subjected to culture on differentiating conditions for 2 more days before fixation and stain. Ch=chitosan; P(OTE)= poly(OTE).

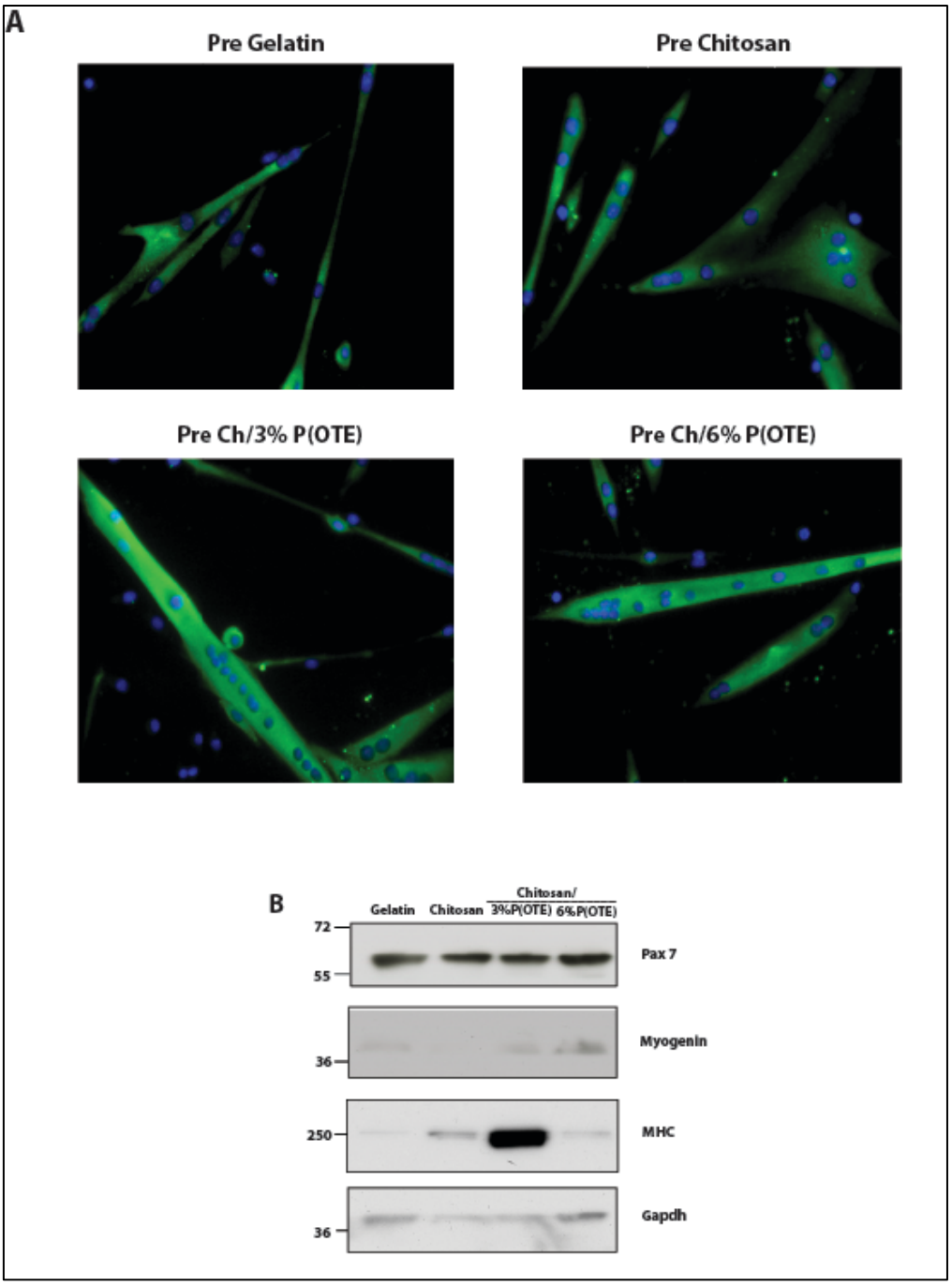

Figure 21. Determination of pMbs differentiation potential *in vitro*.
A. 40X representative images of MHC expression detected by immunofluorescence. Myoblasts were cultured on polystyrene culture plates for 5 days on differentiation media after their culture on different polymer films for 2 days. N=2. Myotubes were fixed and immunostained with an anti-MHC antibody. Nuclei were counterstained with DAPI. **B.** Westernblot of Pax7, myogenin and MHC expression after 5 days on differentiation media. Gapdh was used as a loading control. PRE= Pre cultured on. Ch=chitosan; P(OTE)= poly(OTE).

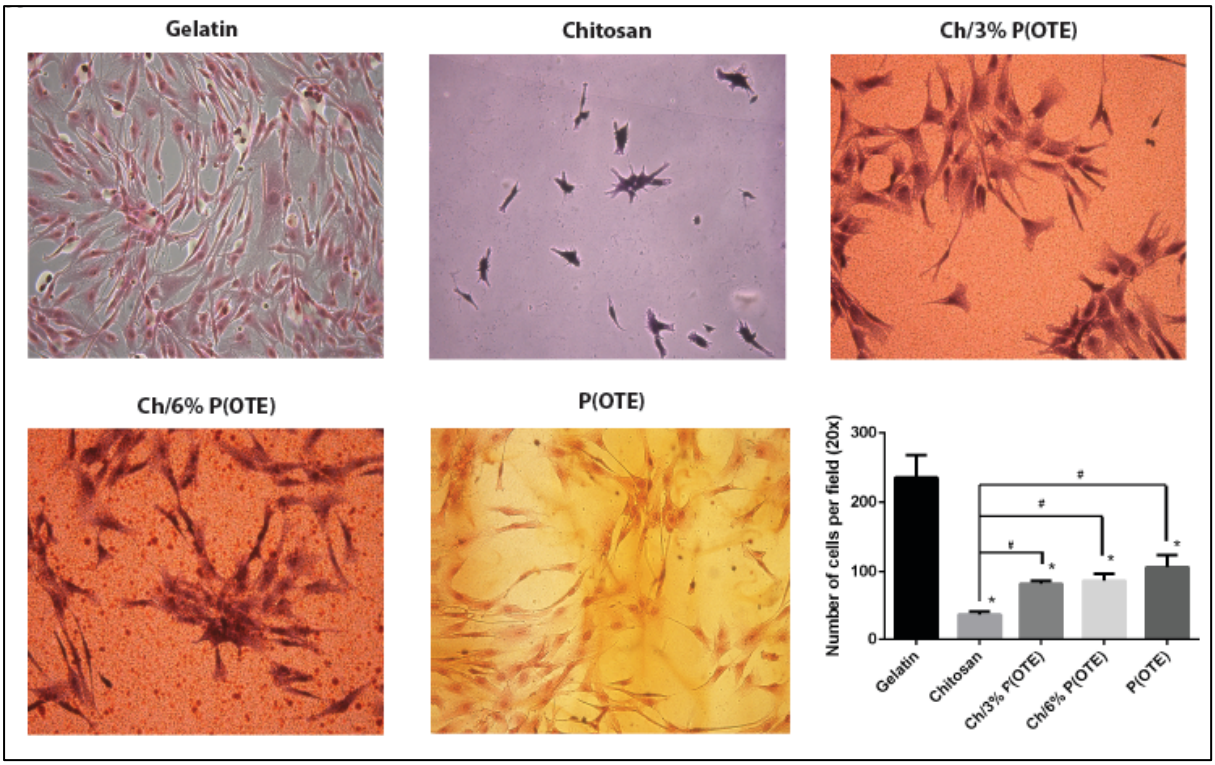

Figure 22. hMbs culture on all polymer films in proliferative conditions.
20X representative images of hMbs cultured on each polymer film on proliferation conditions and images quantification. Cells were cultured on proliferation media for 3 days (in duplicate) for each polymer film then fixed and stained with hematoxylin-eosin. Error bars represent standard deviation counts from ten images for each duplicate sample. One-way ANOVA, *denotes significant difference ($P < 0.05$) with control Gelatin; # denotes significant difference ($P < 0.05$) with chitosan. Ch=chitosan; P(OTE)= poly(OTE).

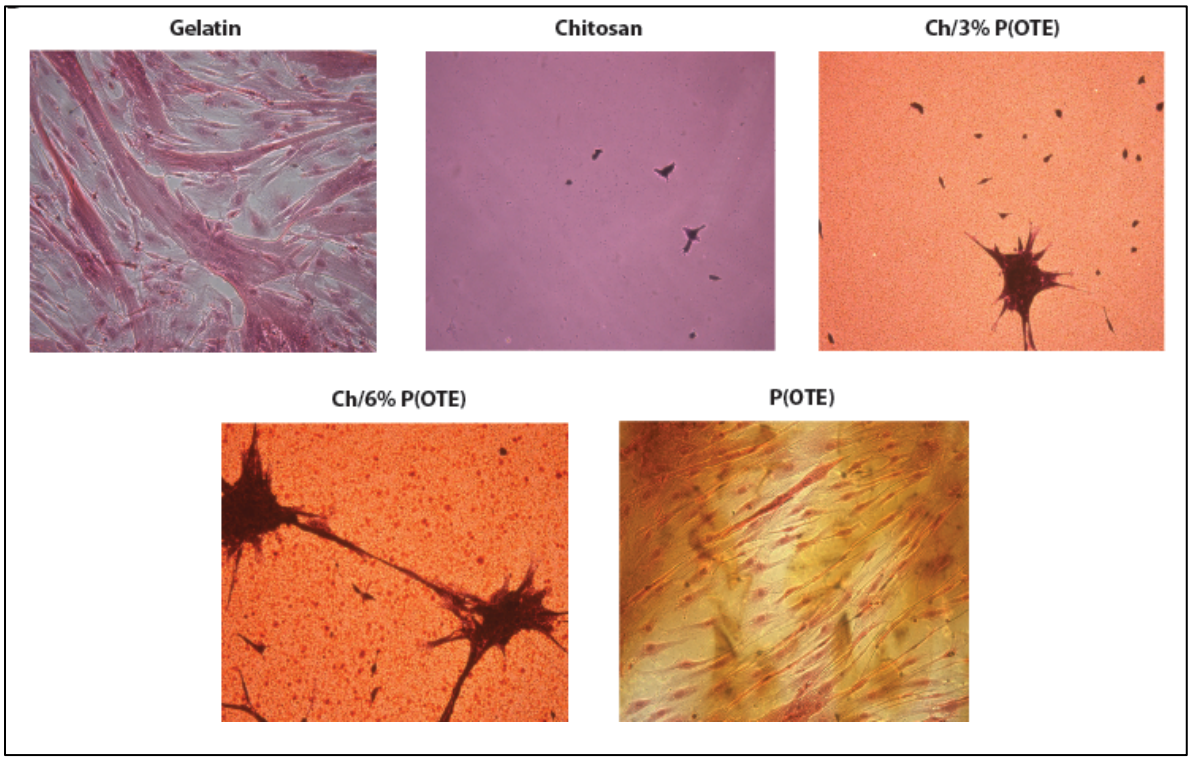

Figure 23. hMbs culture on all polymer films in differentiation conditions**.**
20X representative images of hMbs cultured on each polymer film on differentiation conditions. Cells were cultured on proliferation media for 3 days (in duplicate) for each polymer film and then subjected to culture on differentiating conditions for 4 more days before fixation and staining. Ch=chitosan; P(OTE)= poly(OTE).

7 DISCUSSION

Chitosan/poly(OTE) films showed the formation of an heterogeneous blend with poly(OTE) domains dispersed through chitosan without chemical interaction between this two polymers. Chitosan/3% poly(OTE) blends had more homogeneous distribution of poly(OTE) domains than 6% poly(OTE) blends, probably because the method used was not efficient for higher concentrations of poly(OTE). To be certain, other methods should be explored (*e.g.,* sonication) to obtain more homogenous and reproducible emulsions. In spite of this, cell culture results on these blends were very consistent so maybe these differences are not so crucial to affect myoblast behavior.

Protein adsorption assays showed no differences between the different polymer films, suggesting that this may not be a relevant factor that affects myoblast biocompatibility on the polymers studied. This assay only considered total albumin (main protein present on FBS), a globular protein that appears not to be as relevant to cell-biomaterial interactions, unlike other proteins such as vibronectin and fibronectin that play more important roles on this phenomena (Wilson et al., 2005). Other methods to determine protein adsorption should be assed, for example the detection of adhesion proteins on polymer surfaces by Western Blot.

In vitro culture of mouse myoblasts on chitosan and chitosan/poly(OTE) blends induced a change on myoblast morphology in the case of C2C12 cells and an increase of Pax7 expression levels on both cell types. However, the presence of poly(OTE) in chitosan suggests an increase on myoblast adhesion, which is very important for our desired application, since it is necessary to obtain a sufficient number of functional myoblasts for cell transplant.

We observed an increase on Pax7 expression without an increase in MyoD levels on chitosan/poly(OTE) blends. Pax7 is a marker that is expressed on the first stages of myogenic progression and MyoD is one of the earliest markers of myogenic commitment (Asakura et al., 2007). Thus, myoblasts cultured under these blends appear to maintain a committed myogenic fate.

Differentiation assays exacerbated C2C12 myoblasts morphology. This elongated morphology is characteristic of stress fibers, for example, when increasing the substrate stiffness on cell culture (Yeung et al., 2005). Studies have suggested that muscle differentiation is strongly influenced by substrate stiffness: C2C12 myoblasts only showed striations characteristic of maturation on a small range of soft gels or on top of a layer of myotubes of approximately equal stiffness (Engler et al., 2004). Also, myoblasts need stiffer substrates for proliferation than differentiation (Boonen et al., 2009). On the other hand, the fact that these cells formed clusters when induced to differentiate suggests that they maintain their migration capability but they cannot proceed to fusion and terminal differentiation. Also MyoD expression is increased on differentiation conditions on chitosan/poly(OTE) films compared to PS control. Despite MyoD expression, terminal differentiation did not proceed. We speculate that this is due to the increase in Pax7 levels, since it has been shown that Pax7 represses MyoD function, which is critical to prevent precocious differentiation (Olguin & Olwin, 2004; Olguin et al., 2007). Remarkably, both cell types are able to maintain their differentiation capacity, since robust myotube formation was observed after transferring chitosan/poly(OTE) cultured cells to a conventional substrate (*e.g.,* gelatin or PS). These findings are very interesting and could be very useful for skeletal muscle engineering. Most studies have focused on the development of new polymeric scaffolds for myoblast transplantation, however, for the success of these strategies, the ability to obtain a sufficient number of myoblasts prior transplantation is critical. This can be obtained by *in vitro* expansion of autologous satellite cells, but once plated, the self-renewal and regenerative capacity of myoblasts is rapidly lost, characterized by a rapid and significant decline of Pax7 expression and a significant increase in the expression of MyoD (Ding et al., 2015) obtaining an insufficient number of functional progenitor cells. Because chitosan/poly(OTE) blends had shown to stimulate and maintain myoblasts Pax7 expression, they could be a suitable scaffold to fulfill these requirements.

We observed significant differences between cultures of C2C12 and pMbs on poly(OTE) films. The biocompatibility trend was opposite for each cell type, with higher cell number in the case of C2C12 and lower for pMbs. Poly(OTE) films effect on C2C12 has already being described obtaining similar results. However, our results for pMbs are different from previously reported (Breukers et al., 2010). They observed a big cellular density and formation of myotubes, while we observed almost no cell adhesion in these experiments. This difference may be due to poly(OTE) doping methods. Our poly(OTE) was doped with ion chloride while Breukers et al. (2010) used para-toluene sulphate acid (pTS). pTS is bigger than chloride, so it can affect poly(OTE) chains arrangement. These findings highlight the importance of using the right cell type for *in vitro* studies, in order to determine if there are big differences between cell lines, primary cultures and cells from different species to select a suitable cell model for studies on a particular polymer scaffold. Preliminary studies using hMbs showed that these cells behave very similar to C2C12 myoblasts, rather than pMbs, which was interesting since C2C12 is an immortalized myoblast cell line gene and protein expression. These findings showed that on chitosan/poly(OTE) blends, C2C12 cells are a better cell model than pMbs for *in vitro* studies of human muscle regeneration, with chitosan/poly(OTE) blends.

Although the beneficial effects of the *in vitro* culture of myoblasts on chitosan/poly(OTE) blends are clear, the mechanisms of why these blends maintain myoblast lineage commitment and differentiation potential remains unknown. Films surface studies indicate that the blends present a slight increase of roughness and an heterogeneous poly(OTE) distribution, that could be a factor that contributes to these findings, but there are many other factors that may also contribute to this phenomena. As discussed in earlier chapters, cell response when exposed to a biomaterial is multifactorial. The extent and strength of cell adhesion, and subsequent cell proliferation depend strongly on the physical and chemical properties of the biomaterial surface such as chemical composition, net charge, wettability, conductivity, rigidity and surface roughness among others (Bacakova et al., 2011). Considering our findings, it would be

important to continue chitosan/poly(OTE) films physicochemical characterization to determine if the electroactive characteristic of poly(OTE) could be benefiting myoblast biocompatibility or a decrease of wettability on the films, since this polymer is less hydrophilic than chitosan (Breukers et al., 2010; Noriega & Subramanian, 2011).

Future work should include blends characterization with contact angle measurements and conductivity assays. Cytotoxicity assays (*e.g.,* lactate dehydrogenase measurement) of myoblasts cultured on chitosan and chitosan/poly(OTE) polymer films should be evaluated to compare with normal culture conditions, and further protein expression characterization of myoblast population. Since we had problems with immunofluorescence due to poly(OTE) natural autofluorescence, flow cytometry could be a good alternative to evaluate and characterize myoblasts lineage specific protein expression and to determine possible differences on expression on different adhesion proteins. Finally, it is pending to evaluate the ability of myoblasts cultured on these polymers to integrate to existing muscle tissue and contribute to regeneration in a mouse model, using GFP-labeled myoblasts and fluorescence image analysis.

8 CONCLUSION

Our results suggest that chitosan/poly(OTE) blends improve chitosan myoblast adhesion and could be a suitable substrate/scaffold to maintain myoblasts on an early stage of the myogenic lineage, retarding differentiation prior to transplantation due to an increase of Pax7 expression levels, while C2C12 cells could be a suitable cell model for future human studies with chitosan/poly(OTE) blends, since preliminary hMbs culture studies showed similar behavior. Therefore, this polymer blend shows potential for applications in myoblast-based regenerative medicine and skeletal muscle engineering. Future studies include to complete blends characterization, and optimization. Also cytotoxicity assays are pending in order to continue with *in vivo* studies.

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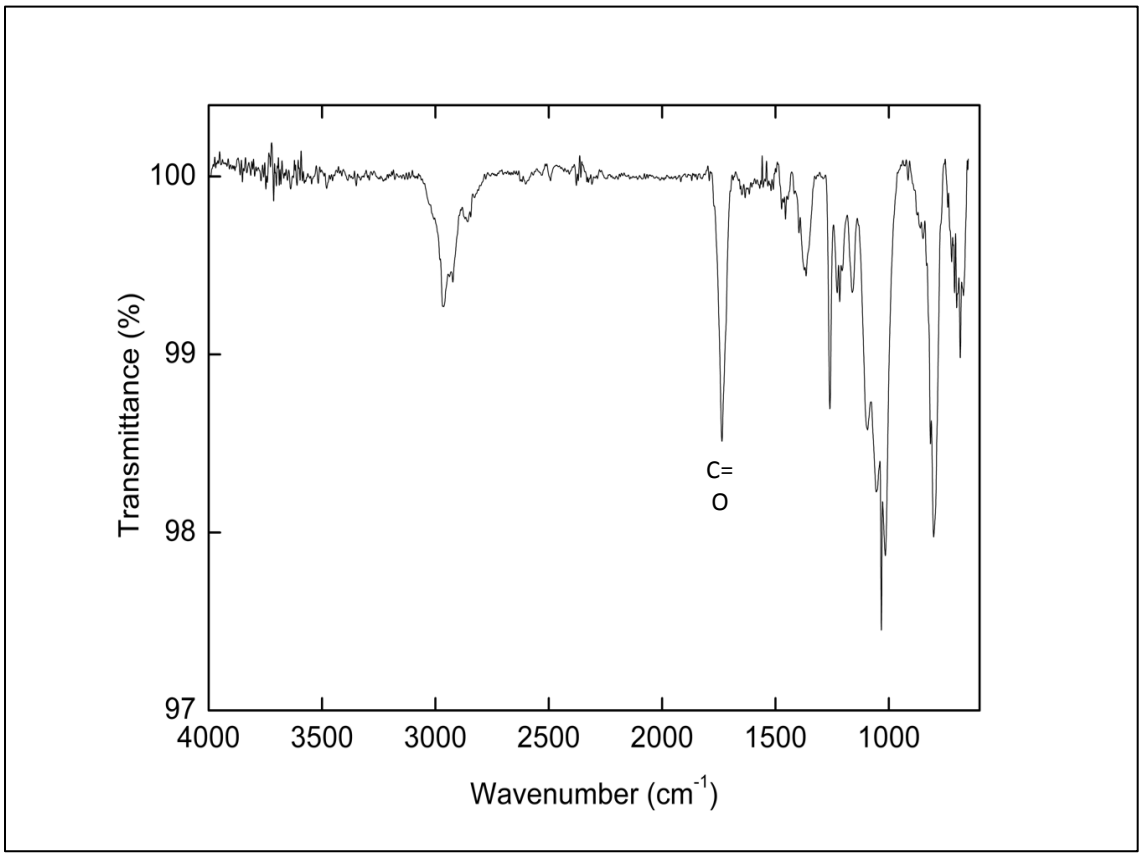
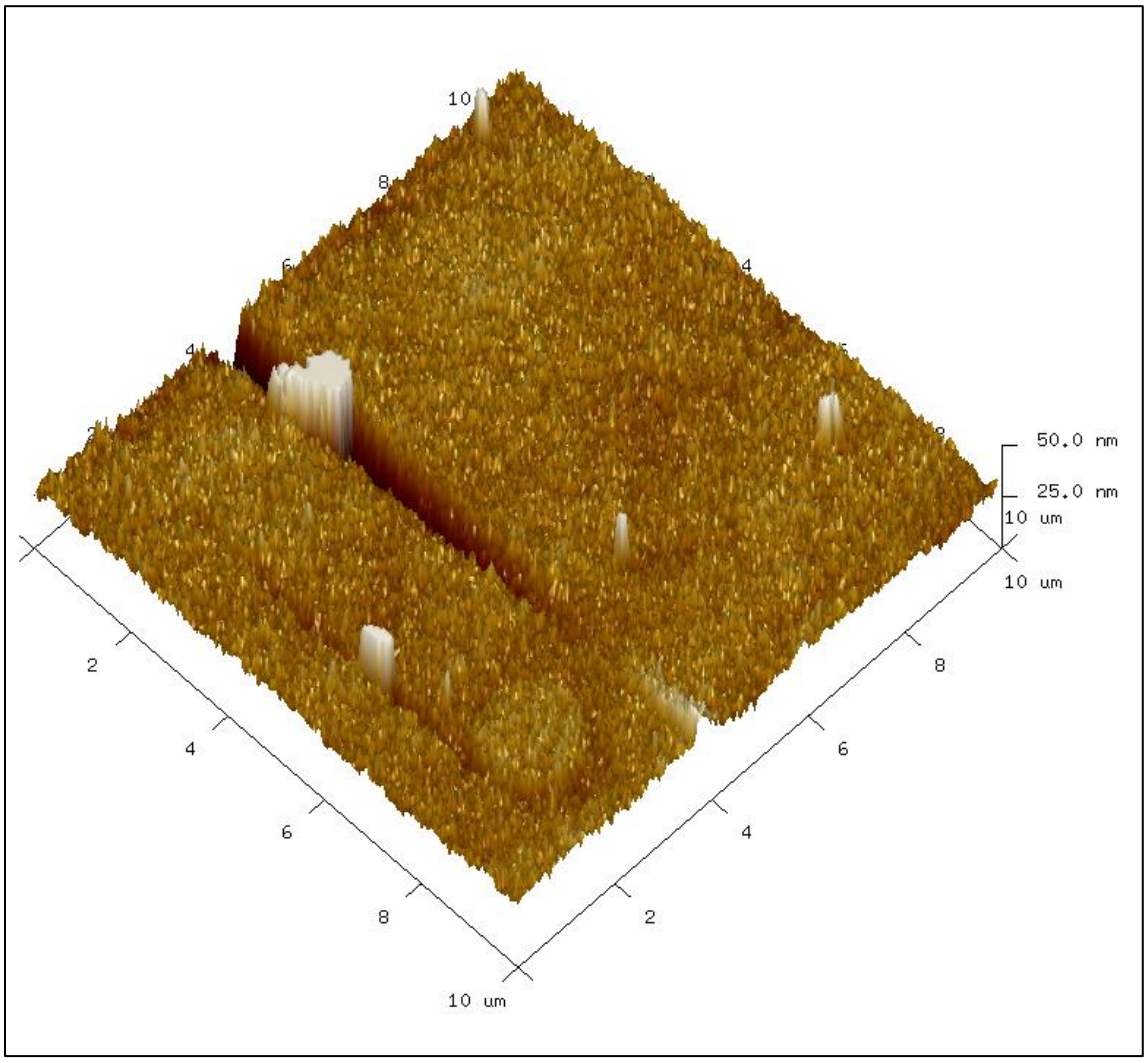
10 ANNEXES

ANNEX A



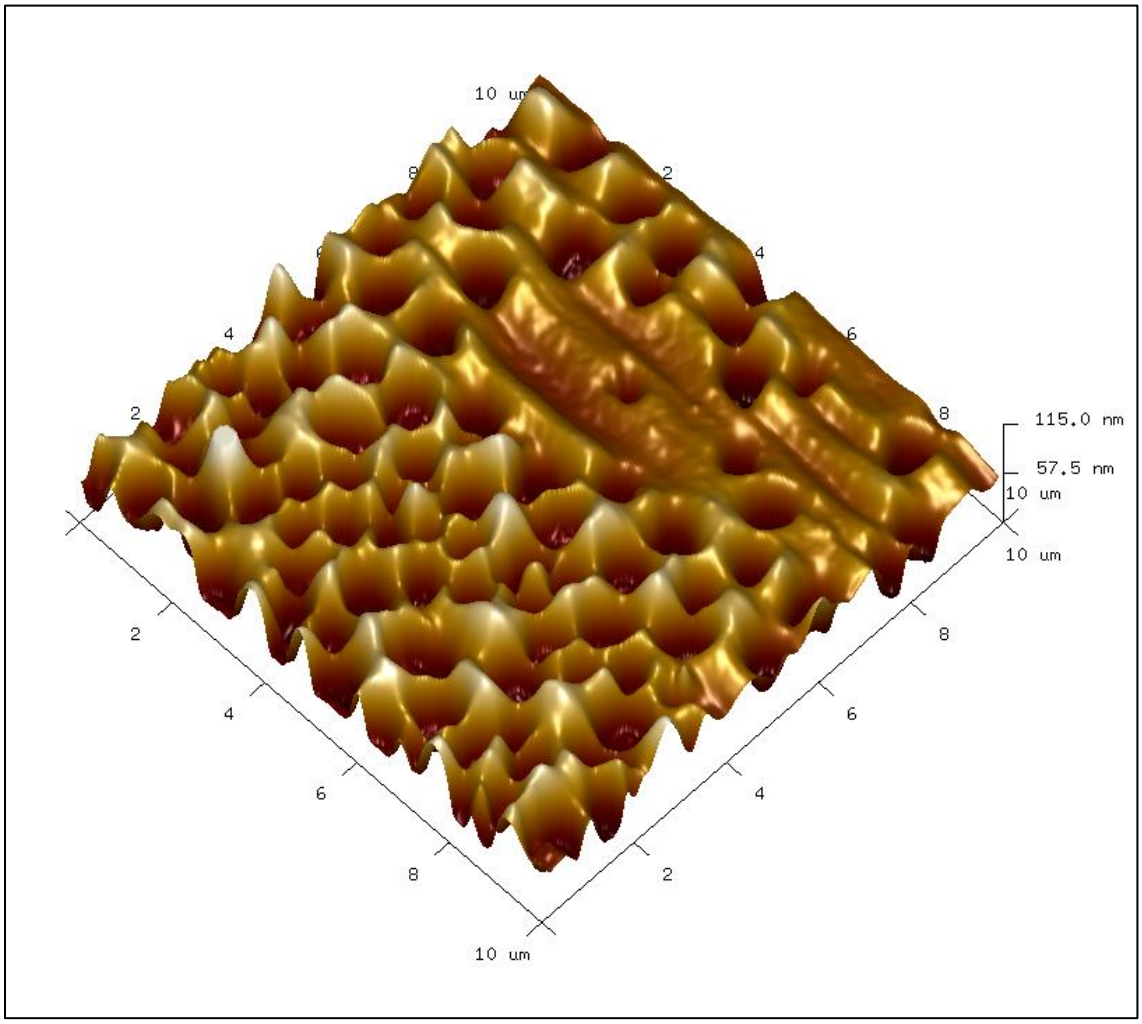
FT-IR spectrum of poly(OTE).
The spectrum show characteristic signals of carbonyl group (C=O) at 1736 cm^{-1}, C-H aliphatic stretching at 2923 and 2859 cm^{-1}, and C-O-C symmetric and asymmetric stretching between 1230-1000 cm^{-1}. Poly(OTE) presence is evidenced by a small signal at 640 cm^{-1} that corresponds to C-S-C stretching from the polymer's thiophene ring. Also, a small signal at approximately 800 cm^{-1} is present, corresponding to the trisubstituted thiophene ring (position 2, 3, 5).

ANNEX B



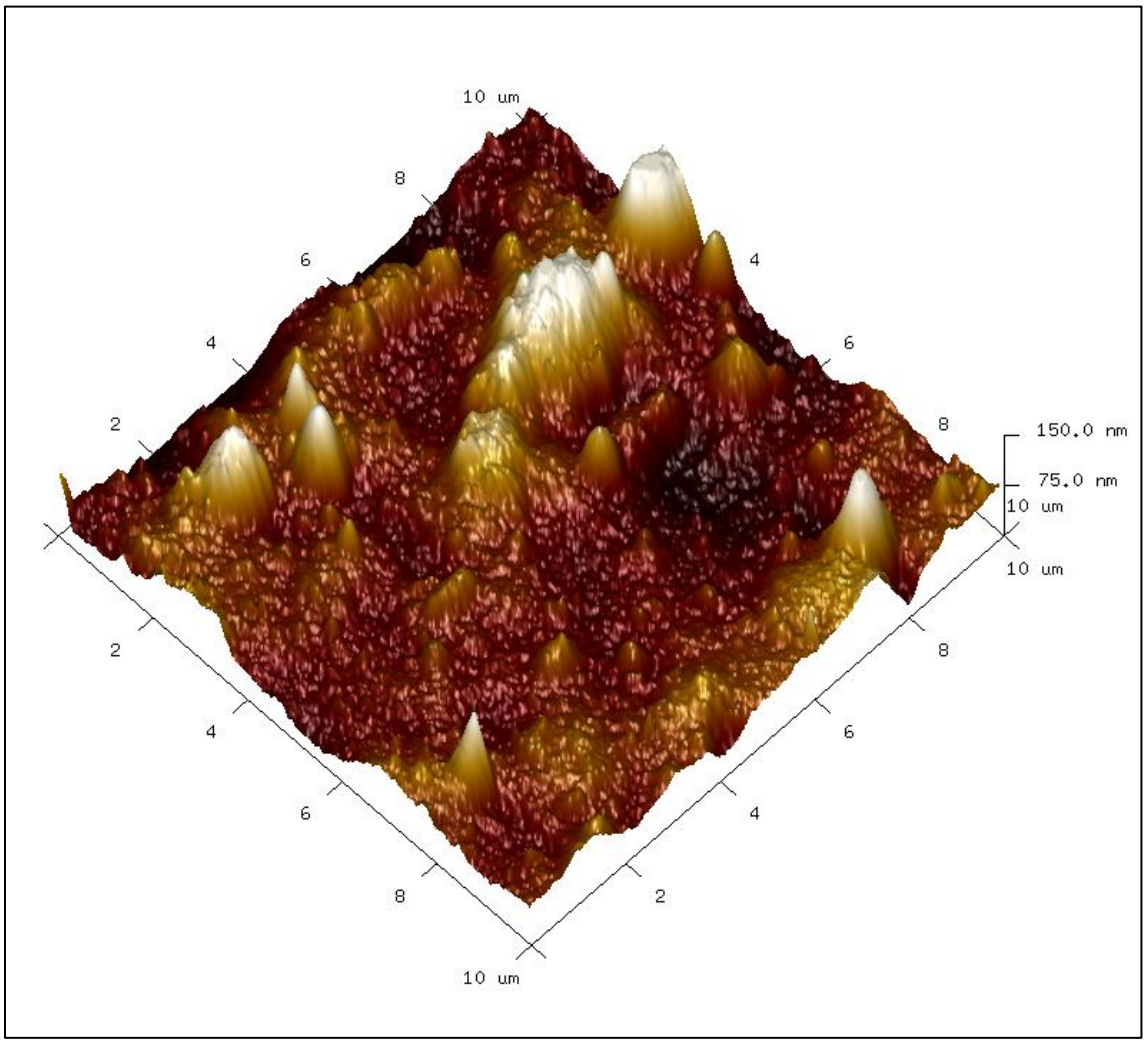
AFM 3D topographic image of Chitosan film.
The film was scanned over 10 X 10 µm area.

ANNEX C



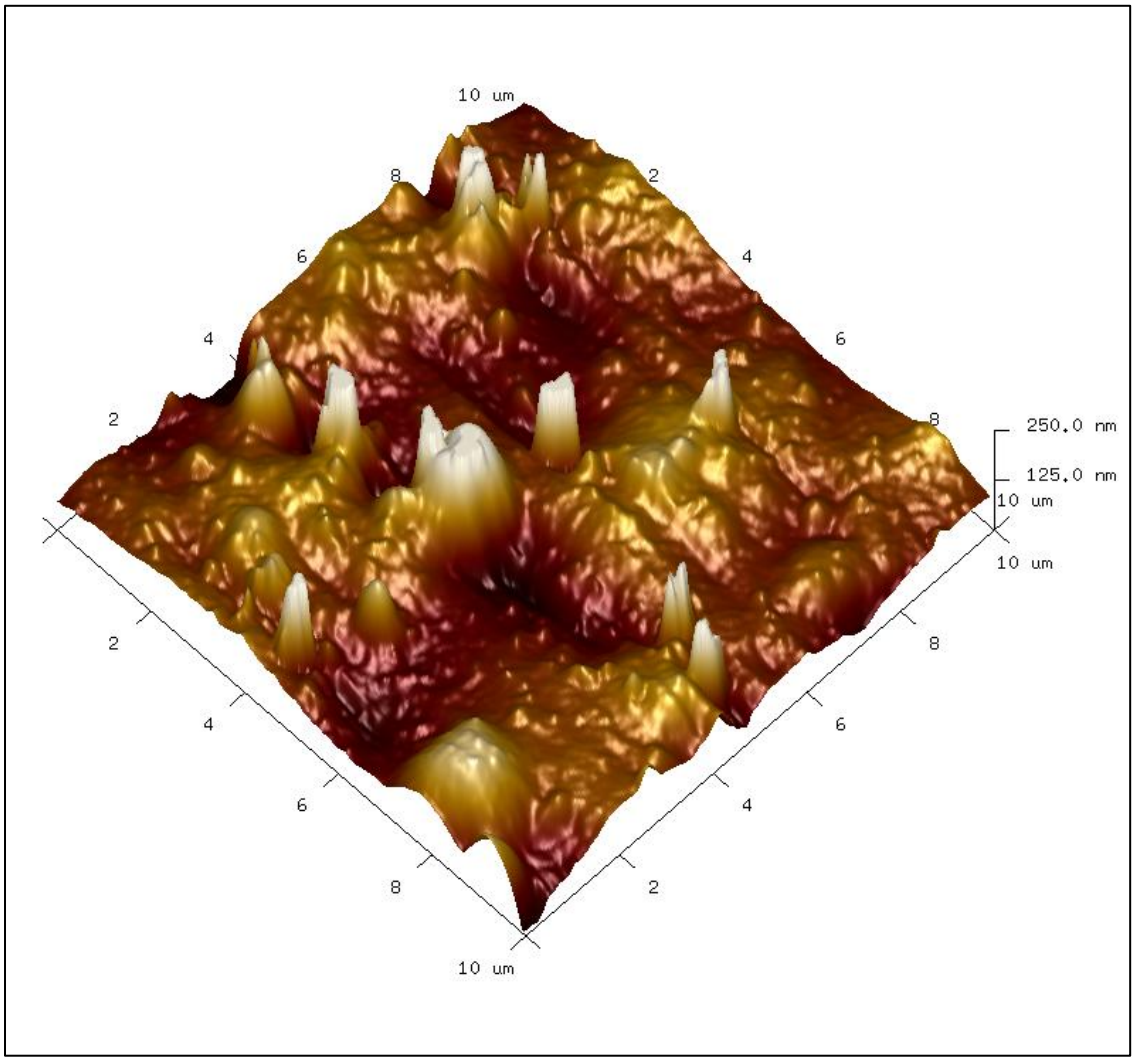
AFM 3D topographic image of poly(OTE) film.
The film was scanned over 10 X 10 μm area.

ANNEX D



AFM 3D topographic image of Chitosan/3% poly(OTE) film.
The film was scanned over 10 X 10 μm area.

ANNEX E



AFM 3D topographic image of Chitosan/6% poly(OTE) film.
The film was scanned over 10 X 10 µm area.

ANNEX F

DOCUMENTO DE CONSENTIMIENTO INFORMADO

Protocolo Clínico	**Obtención de Muestras de Tejido Muscular Esquelético Humano para cultivo celular**
Nombre del Estudio:	Relevance of β4 integrin as a marker of a potential intermediate progenitor after satellite cell activation in the perspective of cell-based therapies for Muscular Dystrophies/ Relevancia de integrina β4 como marcador de una población celular de progenitores intermedios, posterior a la activación de células satélite, en la perspectiva de terapias celulares para las distrofias musculares.
Patrocinador del Estudio / Fuente Financiamiento	Escuela de Medicina y Facultad de Ciencias Biológicas de la Pontificia Universidad Católica de Chile/ Financiado por FONDECYT
Investigador Responsable:	Dr. Juan Carlos Casar Leturia
Depto./UDA	Depto. De Neurología, Escuela de Medicina, Pontificia Universidad Católica de Chile
Teléfono	2354 3316
Co-investigadores	Dr. Luis Irribarra Trivelli, Depto. De Traumatología y Ortopedia, Escuela de Medicina, Pontificia Universidad Católica de Chile Dr. Hugo Olguín Marín, Depto. De Biología Celular y Molecular, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile

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El propósito de esta información es ayudarle a tomar la decisión de participar o no en una investigación médica.

Tome el tiempo que requiera para decidirse, lea cuidadosamente este documento y hágale las preguntas que desee al médico o al personal del estudio.

Este estudio está siendo financiado por FONDECYT, quien provee de fondos a los investigadores a cargo del estudio y a la institución –la Escuela de Medicina y el Departamento de Biología Celular y Molecular de la Facultad de Biología de la Pontificia Universidad Católica de Chile-, para la realización del mismo.

OBJETIVOS DE LA INVESTIGACIÓN

Usted ha sido invitado/a a participar en este estudio porque será sometido a una intervención quirúrgica traumatológica indicada por su médico tratante, durante la cual, por motivos técnicos, se obtienen muestras de tejido muscular que deben ser desechados.

Solicitamos su autorización para utilizar este tejido muscular en investigaciones sobre temas relacionados a la regeneración muscular, a las propiedades de cultivo de las células musculares y su potencial uso en tratamientos de trasplante para enfermedades musculares.

Estamos interesados en evaluar las condiciones de cultivo fuera del cuerpo humano de las células musculares, la existencia de marcadores celulares que favorezcan su cultivo y que las hagan potencialmente útiles para tratamientos de trasplante a personas con diferentes enfermedades musculares y estudiar genes relacionados con la función muscular en personas sanas y enfermas.

PROCEDIMIENTOS DE LA INVESTIGACIÓN:

Se le pedirá que lea con cuidado y sin apuro este documento.

Sólo si usted ha entendido el propósito y los eventuales riesgos y beneficios de esta investigación médica, y se le han respondido todas sus dudas, por favor firme este documento de consentimiento.

Su decisión de participar o no en el estudio no afectara la realización del procedimiento quirúrgico al que será sometido/a ni los cuidados que Ud. recibirá por parte del equipo médico.

Una copia firmada de este documento se le entregará a usted.

Si decide participar del estudio, las muestras de tejido muscular obtenidas durante el procedimiento quirúrgico (cuya cantidad debiese ser menor a una cucharada de té) serán recolectadas en un tubo estéril con solución fisiológica y enviadas al laboratorio del Dr. Hugo Olguín M., profesor de la Facultad de Ciencias Biológicas de la Pontificia Universidad Católica de Chile, donde serán procesadas y almacenadas en forma indefinida. Las muestras obtenidas serán manejadas en forma anónima.

De estas muestras se obtendrán células musculares para ser mantenidas en cultivo o congeladas y almacenadas para su uso posterior. Las células en cultivo serán estudiadas bajo distintas condiciones, y podrán ser usadas para el estudio de genes relacionados con la función y/o la regeneración muscular en personas sanas y enfermas. No está planificado realizar trasplante de células a pacientes en esta etapa de la investigación.

Las células que se congelen serán almacenadas en forma indefinida en el laboratorio del Dr. Hugo Olguín, en la Facultad de Ciencias Biológicas de la Pontificia Universidad Católica de Chile.

Las muestras podrán ser utilizadas en otros proyectos de investigación relacionados, bajo las mismas condiciones explicitadas en este documento. Si en el futuro son usadas para propósitos diferentes a los descritos, se le solicitará un nuevo consentimiento.

Por estas razones no existe un número predefinido de muestras a recolectar. El número total final de pacientes a los que se solicitará donar muestras de tejido muscular se definirá según los resultados obtenidos en el laboratorio.

BENEFICIOS:

Usted no se beneficiará por participar en esta investigación médica. Sin embargo, la información que se obtendrá será de utilidad para conocer más acerca de los mecanismos de

regeneración muscular en humanos y eventualmente podría beneficiar a otras personas con enfermedades musculares.

RIESGOS

Participar en esta investigación médica no tiene riesgos para usted. Los riesgos asociados a su cirugía serán explicados a Ud. por su médico tratante.

COSTOS:

Participar en este estudio no tendrá costos extras para Ud. Los costos derivados de su hospitalización y cirugía no son cubiertos por el estudio y son de responsabilidad del paciente o su seguro de salud.

COMPENSACIONES.

No está contemplado realizar compensaciones por participar en este estudio.

CONFIDENCIALIDAD DE LA INFORMACIÓN.

La información obtenida se mantendrá en forma confidencial. Es posible que los resultados obtenidos sean presentados en revistas y conferencias médicas, sin embargo, su nombre no será conocido.

VOLUNTARIEDAD

Su participación en esta investigación es completamente voluntaria. Usted tiene el derecho a no aceptar participar o a retirar su consentimiento en el momento que lo estime conveniente. Al hacerlo, usted no pierde ningún derecho que le asiste como paciente de esta institución y no se verá afectada la calidad de la atención médica que merece.

Si usted retira su consentimiento, las células obtenidas de sus muestras serán eliminadas y la información obtenida no será utilizada.

PREGUNTAS:

Si tiene preguntas acerca de esta investigación médica puede contactar o llamar al Dr. Juan Carlos Casar, Investigador Responsable del estudio, al teléfono 2354-3316.
Si tiene preguntas acerca de sus derechos como participante en una investigación médica, usted puede llamar a la Dra. Beatriz Shand K., Presidente del Comité de Ética en Investigación de la Escuela de Medicina, Pontificia Universidad Católica de Chile, al teléfono 2354-8173, o al mail: etica.investigacion@med.puc.cl.

DECLARACIÓN DE CONSENTIMIENTO.

- Se me ha explicado el propósito de esta investigación médica, los procedimientos, los riesgos, los beneficios y los derechos que me asisten y que me puedo retirar de ella en el momento que lo desee.
- Firmo este documento voluntariamente, sin ser forzado a hacerlo.

- No estoy renunciando a ningún derecho que me asista.
- Se me ha informado que tengo el derecho a reevaluar mi participación en esta investigación médica según mi parecer y en cualquier momento que lo desee.
- Al momento de la firma, se me entrega una copia firmada de este documento.

FIRMAS

- **Participante**

  Nombre……

  Firma…… Edad……..

  Fecha……..

- **Investigador Responsable**

  Nombre……

  Firma……Fecha……..

- **Director de la Institución o su Delegado**

  Nombre……

  Firma……Fecha……..