Development of polymeric fibers by centrifugal spinning process and their potential to adhere and proliferate human fibroblast cells

Desarrollo de fibras poliméricas por el proceso de hilado por centrifugación y su potencial para la adhesión y proliferación de fibroblastos humanos

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Abstract

Since regenerative medicine is highly dynamic and it is under continuous evolution, one of the challenge points to the development of new materials is the potential to mimic the extracellular matrix (ECM) and biocompatibility within living organisms. The main objective of this study is the development of polymeric fibers based on mixtures of Polylactic Acid (PLA), Carboxymethylcellulose (CMC) and Chitosan (CH) of different molecular weights through the centrifugal force spinning process (CSP). The morphology, the diameter, as well as the migration of JB/3T3 human fibroblast cells on the obtained microfibers were evaluated by scanning electron microscopy (SEM). Biological determinations, such as cell adhesion and proliferation, were evaluated by the MTT assay at 24 and 144 h. These tests were used as functionality and biocompatibility parameters of the obtained meshes. The results showed that CSP is a good method to produce well-shaped and handle-resistant fibers with diameters not exceeding 8 μm. In addition, the results of cell adhesion and proliferation indicated that the viability in the fibers improved by up to 25% after the evaluation hours, which suggests that the polymer mixtures used could benefit fibroblast cell migration and recreation of the MEC. In the case of the arrangement and diameter of the fibers obtained, it is suggested that this can facilitate the transport of nutrients and cellular waste. Finally, the results obtained indicate that CSP is an efficient method to produce microfibers on a large scale with possibilities of being used within the biomedical area.

Keywords: Biocompatibility, cell adhesion, cell viability, copolymers, fibers, centrifugal spinning.

Resumen

El desafío actual apunta al desarrollo de nuevos materiales con el potencial de recrear la matriz extracelular (MEC) y que posean una alta biocompatibilidad dentro de los organismos vivos. Se presenta el desarrollo de fibras poliméricas a base de mezclas de Ácido Poliláctico (PLA), Carboximetilcelulosa (CMC) y Quitosano (CH) de diferentes pesos moleculares mediante el proceso de hilado por fuerza centrífuga (CSP). La morfología, el diámetro, así como la migración de células de fibroblastos humanos JB/3T3 sobre las microfibras obtenidas se evaluaron mediante microscopía electrónica de barrido (SEM). Las determinaciones biológicas, como la adhesión y proliferación celular se evaluaron por el ensayo MTT a 24 y 144 h, estas pruebas se utilizaron como parámetros de funcionalidad y biocompatibilidad de las mallas obtenidas. Los resultados mostraron que CSP es un buen método para producir fibras bien formadas y resistentes a la manipulación con diámetros que no exceden las 8 μm. Además, los resultados de adhesión y proliferación celular indicaron que

la viabilidad en las fibras mejoro hasta en un 25% al pasar las horas de evaluación, o que sugiere que las mezclas de polímeros utilizadas podrían beneficiar la migración de las células de fibroblastos y la recreación de la MEC. En el caso del arreglo y diámetro de las fibras obtenidos se siguiere que esto puede facilitar el transporte de nutrientes y desechos celulares. Finalmente, los resultados obtenidos indican que CSP es un eficiente método para producir microfibras a gran escala con posibilidades de ser utilizadas dentro del área de biomédica.

Palabras clave: Biocompatibilidad, adhesión celular, viabilidad celular, copolimeros fibras, hilado por centrifugación.

Introduction

Lately, it has been observed that multiple advances within the area of biomaterials have revolutionized the field of tissue regeneration (TR) (Hélary *et al.*, 2015). For example, the creation of innovative medical approaches based on metallic, polymeric, ceramic, and composite materials have been used to fulfill specific functionalities such as bone substitutes (Wang *et al.*, 2012) , scaffolds for nerve regeneration (Koh *et al.*, 2010), wound dressing for skin repair (Dumville *et al.*, 2013), controlled delivery systems (Zhang *et al.*, 2013) , among others. Due to the fact that the perfect biomaterial does not exist, the common interest lies in the creation of new and smart devices that possess the capacity to generate a positive effect in the final host, such as mimicking ECM, assisting in the repair of an organ/tissue, and complying at the same time, high biocompatibility and zero toxicity, mutagenicity or genotoxicity (Wang, 2013). In this way, polymers have gained recognition among researchers to formulate new biomedical approaches, such as scaffolds, due to their tunable properties which allow them to have vast applications within biomedicine, pharmaceutical, cosmetic, and food, among other research areas (Zhang *et al.*, 2020; Miletić *et al.*, 2019; Ulery *et al.*, 2011) . Ideally, a polymeric device for TR must show ideal chemical composition, physical structure, and mechanical properties to support cell attachment, proliferation, differentiation, and neo-tissue genesis (Koosha *et al.*, 2019; Liu *et al.*, 2012; Chen *et al.*, 2002;) also, tunable degradation rates and nontoxic leachable products give them the necessary attributes to be considered for this type of applications (Ulery *et al.*, 2011).

There are different fiber fabrication technologies with biomedical applications, the most widely used is electrospinning; this is because it allows obtaining fibers on a nano scale with relative ease (Zhou & Gong, 2008). Electrospinning is based on the effect of electrostatic force on materials that are generally polymers, the materials used respond to electrostatic charge by forming a cone that can expel small jets of the material and result in fibers of various sizes, which can even measure a few nanometers (Vaseashta, 2009). By the other hand, centrifugal spinning is a technology based on the production of fibers using centrifugal force rather than electric field forces. Many of the polymers used for the manufacture of nanofibers and microfibers are dissolved in nonpolar solvents, the sensitivity that these can present to the electric field is one of the limitations of electrospinning, and despite the fact that it is a technology widely used for research, the production of fibers at large scale by this method is quite limited (McEachin & Lozano, 2012; Sarkar *et al.*, 2010).

Although biocompatibility of polymeric biomaterials can be easily enhanced by implementing different methods for surface modification (Guney *et al.*, 2013), low mechanical strength and high rates of degradation have directly influenced the low application of certain polymers in TR (Sabir *et al.*, 2009). To overcome these issues, it has been opted for the creation of co-polymers and hybrid materials that exhibit controlled physicochemical and biological functions, while at the same time are able to mimic the hybrid and well-organized polymer nanostructure of the ECM (Watt & Huck, 2013). Natural polymers (gelatin, collagen, chitosan, cellulose, alginate etc.) have advantages over synthetic polymers, such as they may have properties that are specific to attach certain cells, and to ease the production of adhesion proteins or growth factors, also

may being highly biocompatible (Corradini *et al.*, 2017; Li *et al.*, 2005; Chung *et al.* 2002). For these reasons, the aim of this work was to formulate co-polymer fibers based on mixtures of chitosan (CH), carboxymethyl cellulose (CMC), and polylactic acid (PLA) using a barely new method, the centrifugal spinning process (CSP). Physicochemical and biological characterization (including cellular adhesion and cytotoxic parameters) was determined in order to identify the formulation with the adequate adhesion and biocompatibility properties to be considered as a new and applicable alternative scaffold within TR.

Materials and methods

Materials

Chitosan of medium molecular weight (CHMMW), chitosan of low molecular weight (CHLMW), Carboxymethyl cellulose (CMC), Polylactic acid (PLA), chloroform (CHL), Dulbelco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Phosphate Buffer Solution (PBS) pH=7.2, ampicillin-streptomycin solution (1X), trypsin, citric acid, and Sigma-Aldrich cell growth determination kit (CGD1) were acquired from Sigma Aldrich. All chemicals and reagents used were of analytical grade or suitable for cell culture.
BJ/3T3 human fibroblast were purchased from the American Type Culture collection (ATCC, USA), and were cultured in an10% FBS and 1% antibiotic supplemented DMEM in and were maintained at 37 °C in 95% air/5% CO_2 conditions. Culture media was changed every 2-3 days.

Methods

Preparation of fiber solutions

PLA, CH and CMC stock solutions were prepared by dissolving polymers into different solvents. PLA solution was prepared by dissolving the polymer in a 10% (v/v) chloroform solution; then CH and CMC solution were prepared by dissolving powders, independently, in a 4% (w/v) aqueous citric acid solution. All mixtures were stirred at room temperature until polymers were totally dissolved. Then PLA, CMC and CH stock solution were mixed in proportion established in Table 1.

Table 1. Stock and experimental solutions used for the manufacture of fibers by the centrifugal spinning process.

Key	Solution	Concentration
CHLMWI	CHLMW in PLA	2.5% v/v
CHLMWII		5.0% v/v
CHMMWI	CHMMW in PLA	0.5% v/v
CHMMWII		2.5% v/v
CHMMWIII*		5.0% v/v
CMCI*	CMC in PLA	2.5% v/v
CMCII*		3.0% v/v
CMCIII		5.0% v/v

Fiber preparation

About 1.5 mL of experimental solutions were loaded into a Fiberio® Fiberlab® L1000 equipment with a needle of 30G x ½ (PrecisionGlide). The equipment was operated at a controlled room temperature of 18 °C, heat was applied until a stable experimental solutions temperature of 70 ± 1 °C was achieved, and the distance between the needle tip and the collector was fixed at 14 cm. All solutions were spun at 6000 rpm for 5 min, and then spun at 9000 rpm for 1 minute. Fibers were manually collected using a metal frame of 5x5 cm. The membrane obtained was dried at 80 °C to remove traces of the solvent.

Collected fibers were stored in sealed petri dishes at room temperature until used.

Fiber´s characterizations

Scanning electron microscope

Morphology, fiber diameter, and aspect of developed fibers were determined using a scanning electron microscope (SEM; Jeol JSM-6010LA, USA). Fiber diameter distribution was determined through the analysis of micrographs using the JOEL integrated software of the microscope.

Bioactivity evaluations

Cell culture preparation

BJ/3T3 human fibroblast cell line was cultured on 25 cm^2 flask with DMEM supplemented with 10% FBS, 10 units/mL penicillin, 10 µg/mL streptomycin and maintained 24 h in 95% air/5% CO_2 at 37 °C conditions. Cell subculture was carried out when a 75% cell confluence was observed.

In vitro cell culture

Firstly, meshes of developed fibers were cut in 5x5 mm squares and were pre-incubated in a 96-well plate containing supplemented DMEM for about 1 h; then, samples were subjected to sterilization by UV radiation during 20 min. Subsequently, they were washed 10 times with PBS solution containing 10 units/mL penicillin and 10 µg/mL streptomycin. Finally, fibers were rinsed with DMEM containing phenol red until no color change was observed in the medium's color.

Cell viability by MTT assay

MTT assay was performed to determine the cell viability on polymeric devices through the cell growth determination kit (sigma CGD1), following supplier´s instructions. JB/3T3 cells subculture was carried out by enzymatic digestion (trypsin/EDTA solution); then, cells were seeded in triplicate at 10 x 10^4 cells in each well containing pre-treated polymer fiber meshes. Plates were incubated at 37 °C during 24 and 144 h in a 95% air/5% CO_2 conditions. After incubation period, cell medium was removed and MTT solution was added to each well, and plate was incubated for 4 more hours in same mentioned conditions. Cell viability was determined by UV-Vis spectra of formazan complex at 565 nm in a microplate reader; cell growth percentage was determined from the material-response for each selected fiber. All data points were performed in duplicate and repeated independently at least three times for statistical evaluation (mean ± standard deviations).

Results and discussions

Morphology of fibers

Results showed that CMCI, CMCII and CHMMWII generated a mesh with fibers strong enough to be collected and with potential applications for cell adhesion. With the same amount of volume introduced, the mixtures with CHLMW formed thin and fragile fibers that made the collection difficult. In general, the mixtures with CHMMW and CMC allowed the formation of meshes with the obtained fibers. Elongated droplets were found in fibers obtained from CMCI and CMCII (Figs. 1-2).

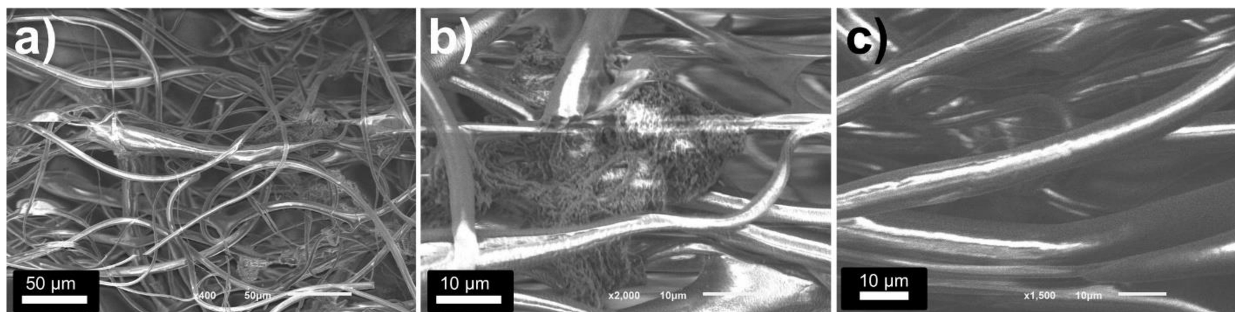

Fig. 1. Morphological evaluations by SEM of fibers produced with mixtures of CMCI. a)50 µm. b)10 µm. c)10 µm.

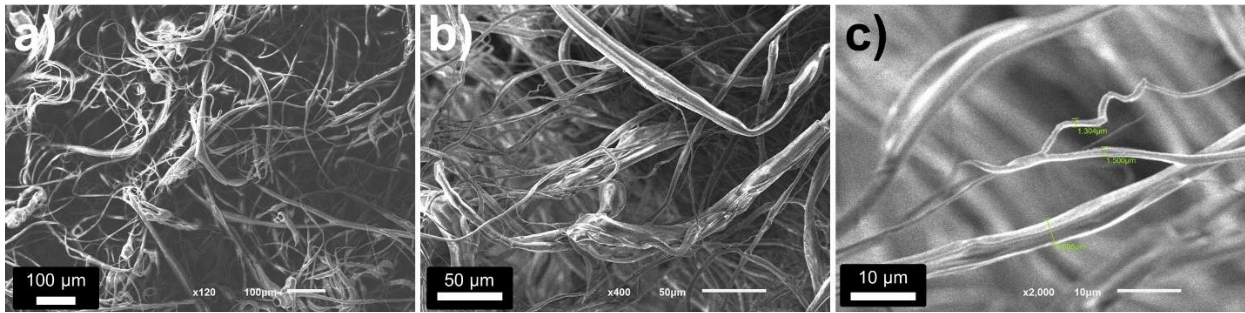

Fig. 2. Morphological evaluations by SEM of fibers produced from mixtures of CMCII. a)100 □m. b)50 □m. c)10 □m.

Under the specified operating conditions, it was observed that at lower concentration of PLA, finer fibers with greater distribution of diameter were found (Fig. 3).

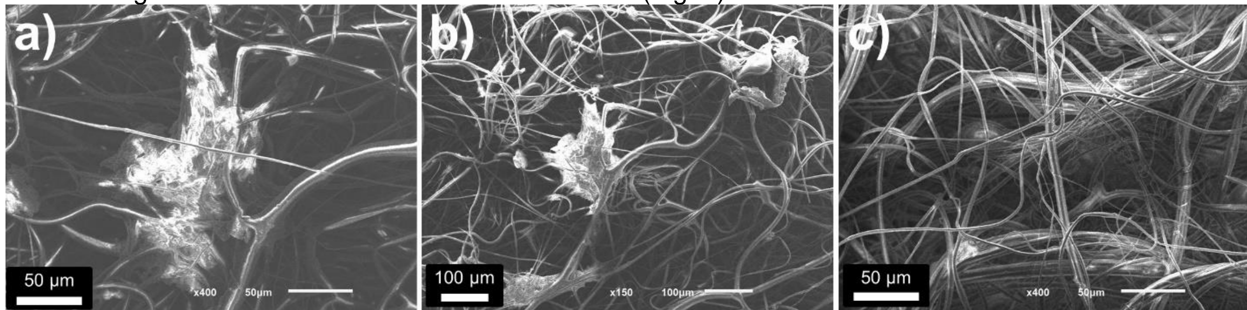

Fig. 3. Morphological determinations by SEM of fibers produced from mixtures of PLA. a)50 □m. b)100 □m. c)0 □m.

Bioactivity determinations

The determinations of the average diameter in produced fibers indicated the following tendency, CMCII> CHMMWII > CMCI. Where the smallest diameter was obtained with the CMCII solution, 2.1 ± 1.4 µm, and the bigger was CMCI 7.9 ± 1.9 µm (Table 2).

Table 2. Average diameter fiber using a Scanning Electron Microscope (SEM).

Sample	Diameter (µm)
CMCI	7.9 ± 1.9*
CMCII	2.1 ± 1.4*
CHMMWII	4.2 ± 1.5*

*All experiments were performed twice in triplicate

Viability assays were carried out to demonstrate the cell adhesion ability and biocompatibility of the developed fibers with different percentages of CMC and CH. Results indicated that after incubation times of 24 and 144 h, JB/3T3 fibroblast can attach to the fiber in the next order: CMCI> CHMMWII > CMCII, as shown in Table 3. It was observed that fibers containing 2.5% of CMC demonstrated to possess the best ability to adhere cells, let them proliferate and differentiate in comparison to those formulations containing ≥ 3.0 % of CC (CMCII) and even better than those fibers containing exclusively CH. Viability assay indicated that CMCI formulations induce more increment in cell proliferation after 3 days in comparison to CMCII. Conversely, according to our findings, those formulations containing concentrations of 5.0 % of CMC presented negative implications in cell adhesion by retarding the fibroblast maturation and proliferation. MTT assay results express a measure of cell

Table 3. MTT assay, % viability of BJ/3T3 fibroblasts.

Sample	% viability (24 h)	%viability (144 h)
CMCI	110.9 ± 13.6*	125.0 ± 11.1*
CMCII	78.6 ± 16.0*	108.4 ± 9.1*
CHMMWII	104.7± 13.8*	109.3 ± 13.4*

*All experiments were performed twice in triplicate

viability from 3T3 cultured onto mat fibers. It was observed that MTT assay values increased during the evaluation time by up to 25%, indicating that the mat of fibers evaluated had no significant cytotoxic effects and could support cell proliferation, since mitochondrial activity is maintained over time.

Discusion

Fibers (at micro and nanoscale) have gained attention for different application, including scaffolds, mainly because they are formed in the same order and diameter, which could enhance their cell-matrix interactions by resembling the ECM environment (Chae *et al*., 2013).

Fibers with lower diameter distribution were found in CMCI (Fig. 1). It has been reported that properties of CH (such as degree of deacetylation) in certain conditions (e.g. solvents, temperature) may frequently hinder the formation of fibers and nanofibers by the electrospinning method (Savitri *et al*., 2014; Nam *et al*., 2010). However, our results showed that the CSP is a good choice for preparing fibers with a chitosan portion in the specified conditions and operating method. These results are of great importance because it has been reported that CSP is a new and alternative method that can be used for producing several types of fibers and nanofibers from various materials at high speed and low cost (Huang *et al*., 2019; Zhang & Lu, 2014).

Also, it was observed that there was a great thickness variability on those fibers obtained with CMCII mixtures. However, stretched droplets were found on the obtained fibers. A possible explanation is that formulations created with 5% of CMC may have excess of COO^- groups, which could generate an acidic environment for fibroblasts (Novotna *et al*., 2013), causing a significant decay ($P \leq 0.05$) in cell viability in comparison to control after 24 h. However, after 3 days of incubation it was observed that cell viability increased, indicating that cells may have adapted to the acidic environment and then proliferated. On the other hand, CHMMWII based formulations demonstrated that induced good cell adhesion after 24 h was achieved, and cell viability slightly increased during 72 h (from 104 to 109 %), which indicate that CH formulations presented a good biocompatibility with fibroblasts. Our findings are in agreement with those studies that have demonstrated that CH has the ability to reinforce the microstructure and/or to modify the surface of medical devices/scaffolds developed at low CH concentrations (≤ 1.0%), creating the ideal microenvironment of porosity for rapid adhesion and cell proliferation (Tan *et al*., 2000).

Conclusion

The main objective of this work was to generate various nanofiber formulations using a not so conventional method, the centrifugal spinning process (CSP). Results indicated that CSP has the ability to produce fibers with diameter that can range between 2 to 9 um, approximately. These diameters and their arrangement may confer different properties that influence the ability to adhere and allow JB/3T3 cells to proliferate. In TR, good biocompatibility of new materials and novel medical devices is often evaluated by *in vitro* cell responses, where results shouldn't indicate any cytotoxicity effect and should provide the adequate surface properties for the cell attachment, proliferation, maturation, and differentiation. Cell viability of JB/3T3 cells was increased 25% when were cultured onto mat fibers, according to these statements, we could suggest that those fiber formulated with 2.5% of CMC and 0.5 % of CH may provide the ideal microenvironment for JB/3T3 cell adhesion. Our results indicate that CSP is a good non-conventional and non-expensive method to produce biocompatible CMC and natural polymer-based microfibers in large scale, that could be used as novel formulations within the tissue regeneration, biomedical products, even drug delivery system field in a near future.

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Interest conflict

The authors declare that does not exist an interest conflict.

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