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Production of Chitosan Microcarriers using Electrospray Equipment

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A public health problem is the loss of tissue due to different etiological factors. Then, a traditional way to rebuild lost tissue is by culturing cells onto the surface of biomaterials. These cultures have been carried out in extended surfaces which do not guarantee the cells growth. Nevertheless, in the last years, the culture over scaffolds arises as a technique which improves momentum, heat, and mass transfer. The biomaterial of these scaffolds is a choice which establishes the good or bad response from the host cells, and the procedure conditions to engineer these scaffolds depend on it. Thus, this manuscript aims to stablish the laboratory conditions to produce chitosan scaffolds. Promptly, it focuses on the study of the conditions to generate microcarriers by using an electrospray equipment, evaluating the physical and cytotoxic characteristics of the microcarriers. The polymer solution was discharged onto a polyphosphate solution for crosslinking the polymer. It was employed a factorial experimental design of three parameters: voltage, polymer concentration and solution flow rate. Microcarriers obtained were collected in a polyphosphate solution, and washed in a Hank´s solution. Microcarriers were analyzed in a light microscope in order to obtain the size distribution. Results evidence a hard relationship between voltage and the mean diameter of the particles, with a none influence from the flow rate nor polymer concentration. The best condition was with a voltage of 17 kV, polymer concentration of 0.5% w/v and solution flow rate of 4.5 mL/h. The mean particle diameter for these conditions was 568.04 ± 81.68 µm. Scanning electron microscopy (SEM) depicts low porosity, which allows the cell culture over the microcarriers surface. Finally, cytotoxicity of the microcarriers was evaluated by using a fibroblasts cell line without adverse effect on the cells. In this way, microcarriers may be used for culture of anchorage dependent cells in spinner bioreactors.

1. Introduction

Traditionally, cells culturing has been realized in 2D employing cells culture flasks, where the cells grow without mixing, presenting no uniform pH and nutrients concentration distributions inside the bioreactor. Thus, the cells growth is limited(Martin et al., 2004). For cell culturing with aim to be used in Tissue Engineering, cellular proliferation must be carried out in 3D environment similar to *in vivo* conditions. It is possible to achieve this in a bioreactor because this technology contributes to increase the concentration per unit volume due to the increment of the rate of cellular proliferation and concentration, diffusion of oxygen to cells, and the possibility of control cellular concentration(Martin et al., 2004; Plunkett and O´Brien, 2011), reducing the culture time and increasing in the homogeneity of the culture medium (Malagón-Romero et al., 2015).
Microcarriers are microspheres obtained using different materials, principally polymers or proteins offer a surface for adhesion and proliferation of anchorage-dependent cells(Lau et al., 2010). There are different materials employed to obtain temporal scaffolds for cells (Pachence et al., 2007; Warren et al., 2004; Malagón-Romero et al., 2015). Addionally, Kim et al. (2005) presented that the use of microcarriers has some advantage as elimination of trypsine to obtain a sheet of cells and less time production. In this way, it is necessary the developing of microcarriers for anchorage-dependent cells. In the literature it was reported that 1 g of microcarriers will cover a superficial area similar to 15 T-flask of 75 cm^2 (Malda and Frondoza, 2006).

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On the other hand, from 2 g of microcarriers it is possible to cover an area near to 2 m^2, which is equivalent to the surface area of human body (Lafrance and Armstrong, 1999).
On the other hand, chitosan is an amino polysacaride obtained at industrial level from n-desacetilation thermo-alkaline of the chitin extracted and purified from crustacean (Hospodiuk et al. 2017). Chitosan is widely used for molding of porous structures, in cell transplantation and tissue regeneration (Karimian et al., 2016),(Radaei et al., 2017),(Hossain et al., 2015). Chitin fibbers and chitosan are biocompatible and do not generate allergic reactions. So, they are recommended for surgery suture because do not need to remove from the wound once it has cicatrized. Along the last decade, chitosan has been widely employed to develop systems for controlled release drug delivery, tissue engineering at laboratory level and, in manufacturing of cicatrizing films at industrial level (Songsurang et al., 2011). Due to cationic behavior and the high density of charge, chitosan is able to adhere to surfaces with a negative charge so it is classified as a bio adhesive material (Zhang and Kawakami, 2010),(Wu et al., 2011).
Chitosan microspheres have been produced from entanglement of chitosan with tri-polyphosphate (Radaei et al., 2017). In this way, Chen et al. (2006) produced chitosan microcarriers with diameter in the range 100-200 μm for murine and human fibroblasts cultures. Maeng et al (2010) have developed microcarriers from chitosan by using an electrospray device, microspheres have a diameter of 150 μm. Wu et al (2011) obtained microspheres with a diameter of 400 μm using chitosan dissolved in acetic acid discharged in sodium tripolyphosphate saturated solution. Radaei et al.(2017) obtained microcarriers with a diameter of 350 μm from a mixture of gelatin and chitosan deposited into a tripolyphosphate solution. This work aims to find standard conditions to obtain microcarriers with a diameter in the range 150-600 μm employing an electrospray equipment.

2. Materials and Methodology

2.1 Preparation of solutions

Chitosan (Sigma-Aldrich, medium molecular weight) was dissolved in acetic acid 100 mM (Sigma-Aldrich, 99.7% purity) at two different concentrations 1.0 % y 0.5 % w/v. For increasing the dissolution of the polymers in the solvent, solution was submitted to ultrasound in an equipment (ATU- ATM40-2LCD) at a frequency of 50 Hz for 10 minutes at room temperature. Solutions were kept at 5 °C until they were employed according to the experimental design.

2.2 Determining the viscosity and conductivity of polymeric solutions

Viscosity was determined in a Rotational Viscometer (Cannon LV Model 2020) at room temperature. Additionally, conductivity was determined in an multiparametric equipment Hanna HI9829-03041 (Romania).

2.3 Experimental procedure to obtain microcarriers

Microcarriers were obtained in an electrospray equipment. This equipment has a maximum voltage source of 30kV (CZE1000R, Spellman), a syringe pump (KDS100), a high voltage probe (HVP-40DM, Pintek), a nozzle and a collector. The distance between nozzle and collector was maintained in 25 cm. In the collector, a commercial sodium tri-polyphosphate solution of 5% w/v in water was deposited to collect microcarriers.

2.4 Experimental design for microcarriers obtaining

It was analyzed the effect of different variables on the diameter and morphology of the microcarriers. According to the reported in literature (Karimian et al. 2016),(Altobelli et al. 2016) it was evaluated voltage, solution flow and polymer concentration. Table 1 presents the experimental design employed in this work.

2.5 Particle Size Determination

Microspheres obtained by electrospray were washed 5 times with a PBS solution. Once finished the last washed, microspheres were washed with Hank´s isotonic sterile solution (MSDS). A sample of the microspheres was fixed on a slide glass and were analyzed in an inverted microscope, equipped with a digital camera. It was taken 5 photos with 10 X magnification for each test. By using the free software ImageJ (www.rsbweb.nih.gov/ij) it was determined the diameter of the microcarriers. Statistical analysis was performed for determining the mean diameter and standard deviation.

2.6 Characterization by scanning electronic microscopy (SEM).

Microcarriers were cross-linked with glutaraldehyde at 1 % in PBSA solution. Microcarriers were dehydrated in grade series of ethanol (50 %, 70 %, 90 % and 100 %). Once suspended the microcarriers in absolute ethanol, they were drying in a desicattor for 24 h. Samples were sputter coated with gold (108 Auto/SE 7008-

220 Sputter Coater) for 40 s and examined in Scanning Electronic Microscopy SEM (Phenom ProX, Switzerland).

2.7 Determination of cytotoxicity of the microcarriers.

Microcarriers were extended on a petri dish and were sterilized with ultraviolet for 1 h in a laminar flow cabinet. Then, microspheres were suspended in 70 % ethanol for 12 h and were washed twice with PBS sterile until eliminate residual ethanol. Finally, microcarriers were suspended in DMEM and were maintained at 37 °C for 24 h. It was determined the concentration of microcarriers per mL by counting the number of microspheres presents in 10 μL, by employing an invert microscope. Concentration was adjusted to 6 cm^2/mL in the DMEM medium, according to ISO 10993-12. Solution was kept in incubator at 37 °C for 24 h to guarantee extraction of components from the material.

Cytotoxicity evaluation employed murine fibroblasts CRL 476. Fibroblasts were cultured in 96-well microculture plates. Each well was loaded with different volumes of extract; control assay was cells without extract. Viability was determined by MTT(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 72 h. 10 μl of MTT was added to each well and was incubated for 4 h at 37 °C. Supernatant was removed and formazan crystals were extracted with 200 μl of DMSO (di-methyl sulphoxide). Absorbance was determined for each well at 570 nm in ELISA equipment. Cellular viability was compared for each well vs the negative control (cells without extract). This test was developed by triplicate.

Table 1: Experimental design parameters

Test	Flow (ml/h)	Voltage (kV)	Solution concentration (% w/v)
A	2.5	14	1
B	1.5	14	1
C	3.5	14	1
D	4.5	14	1
E	2.5	14	0.5
F	1.5	14	0.5
G	3.5	14	0.5
H	4.5	14	0.5
I	2.5	17	1
J	1.5	17	1
K	3.5	17	1
L	4.5	17	1
M	2.5	17	0.5
N	1.5	17	0.5
O	3.5	17	0.5
P	4.5	17	0.5

3. Results and Discussion

Viscosity of the solutions were 66.4 ± 2.1 cP for 0.5% w/v and 321 ± 3.2 cP for 1.0% w/v concentration. Pancholi et al. (2009) reported for chitosan solutions at 0.5% and 2% w/v, a range of viscosities from 40 cP to 825 cP, Similarly, Zhang and Kawakami (2010) reported 32.4 cP for 1.5% w/v solution. The results obtained were in the range reported in the technical sheet published by Sigma-Aldrich (200 – 800 cP for 1% w/w in 1% v/v acetic acid). So the viscosities obtained were similar to reported.

On the other hand, conductivity obtained was 7.67 μS/cm for 0.5% chitosan solution and 14.18 μS/cm for 1.0% chitosan solution. Zhang and Kawakami (2010) reported values of conductivity in the range 200 and 290 μS/cm for chitosan dissolved in acetic acid (90% v/v) for concentrations of 1.5 to 2.0 % w/v, respectively. The difference is due to molecular weight of the polymer and acetic acid concentration.

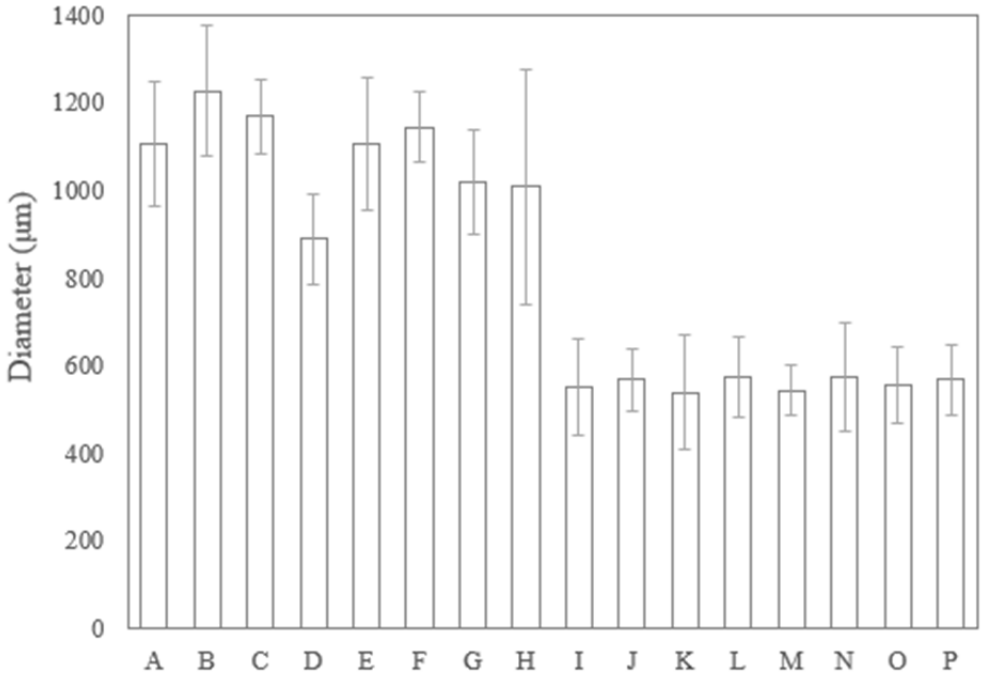

Figure 1. Diameter of the microcarriers obtained experimentally for different experimental conditions.

Maeng et al. (2010) reported production of microcarriers from chitosan employing electrospray but without high voltage, and obtained particles with a diameter upper of 1 mm. The purpose of high voltage is to decrease the diameter of the particles. In this work, Taylor cone was formed in voltages 14 kV – 17 kV, but with a voltage of 14 kV, the electric potential was not sufficient for enabling pulverization of the solution, so, the particles obtained were with high diameter. In the Figure 1, assays A-H correspond to electric potential of 14 kV and assays I-P correspond to electrical potential of 17 kV. When electrical potential was increased, diameter particles decreased. This result was similar to reported in the literature consulted (Radaei et al. 2017), because as voltage increases, the polymer droplet from needle separates more easily and the diameter decreases. In this work the most important variable was electrical potential according to diameter obtained (Figure 1), and the diameter was independent of polymer solution concentration (0.5 w/v and 1.0 w/v) and flow rate (1.5 ml/h – 4.5 ml/h).

The effect of the concentration of the solution is not significant on the diameter. This result is contrary to Maeng et al (2010), who reported that increasing the concentration of the solution from 0.5% to 2.0 %, the diameter of particle also increases from 350 μm to 890 μm. In relation with the flow rate, when the flow rate was increased, diameter of the microparticles will no increase. Similarly, this result is contrary to report (Radaei et al. 2017) who concluded that decreasing the feeding flow rate, resulted in less accumulated drop volume which results in a decreased diameter. These results are due to the concentration solution, because the solution was diluted and the presence of the polymer in solution not affected the volume of the drop.

All of assays were developed at constant distance (25 cm), because if it is employed upper distance, defects in the surface will appear due to impact between particle and solvent. If the distance is less, solvent will not evaporate. Due to considerable effect of electrical potential, we classified in two groups the results obtained: high voltage (assays A-H) and low voltage (I-P). The best conditions correspond to M test ((0.5% w/v, 2.5 mL/h, electric potential 17 kV and distance 25 cm), due to it was the assay with less standard deviation (57.25 μm). The diameter obtained was 543.75 μm, which is upper to reported in the literature consulted (Chen et al., 2006; Maeng et al., 2010; Wu et al., 2011; Radaei et al. 2017). The range reported in the literature is 150 μm – 400 μm, however the diameter obtained it is possible to use for culturing cells in the spinner bioreactor.

By Scanning Electronic Microscopy (SEM) were taken a photo for high and low voltage groups and are shown in Figure 2. According to this Figure, microcarriers surface has some imperfections due to evaporation of acetic acid and low concentration of the chitosan solution employed. Wu et al. (2011) reported that a concentration of 2% w/v to generate more stable microcarriers with possibility to control the shape, size and

aperture. Due to low chitosan concentration, microcarriers volume diminish during dehydratacion with ethanol and finally they had a distinct volume from original suspension, in spite of they were cross-linked with glutaraldehyde at 1%. However, in this work was analyzed the possibility of employing a low concentration of chitosan in order to diminish cost or the microcarriers.

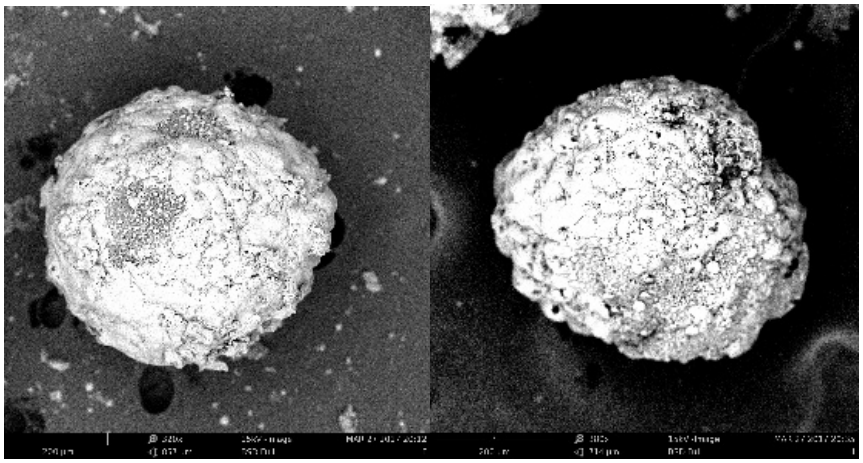

Figure 2: SEM of the microcarriers obtained for low voltage (14 kV, left) and high voltage (17 kV, right).

Viability cellular percentage obtained for cytotoxicity assay was 118.30 ± 16.64% at 72 h. This result is the comparison between absorbance of negative assay (well without microcarriers) and assays (well with microcarriers). This result indicates that microcarriers did not have toxicity for fibroblasts, so it is possible to culture cells in bioreactor on the microparticle surface. Radaei et al. (2017) reported culture of cells for 7 days on the surface of chitosan microcarriers without toxicity detected. According to these result, microspheres may be an ideal candidate to act as carriers for cells or directly applied to the target site (Hossain et al. 2015). In this way, micrrocarriers could be as hemostatic in the wound or injure for patients.

4. Conclusions

This article analyzed the experimental conditions to obtain microcarriers from chitosan by using electrospray. Microcarriers may be used for culturing cells in a spinner bioreactor or directly applied on injure or wound due to low toxicity found. The diameter obtained is higher reported in the literature consulted (543.75 ± 57.25 μm), but is possible to use as scaffold for culturing anchorage-dependent cells. Other alternative is applying on the wound due to hemostatic characteristics offers by chitosan. The low concentration employed for chitosan solution is an additional advance in this work, because the cost of the final product may diminish for patients.

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