Proliferation of 3T3 Fibroblasts in a Spinner Type Reactor on Microcarriers of Fibrin-Alginate

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ABSTRACT- **The microcarriers have become an alternative of cell culture in order to increase the contact area per unit volume of culture medium. The aim of this work is the fabrication of microcarriers from a mixture of sodium alginate and human blood plasma, to be used as a fibroblast support during proliferation in bioreactors. Microcarriers were obtained by spraying through a coaxial nozzle over calcium chloride. Poly-L-Lisine (PLL) and fibrinogen were adsorbed on the surface of microcarriers. The microcarriers were observed by atomic force microscopy (ATM) and scanning electronic microscopy (SEM). Adhesion of 3T3 fibroblasts was evaluated over the microcarriers surface. Microspheres between 150 µm and 500µm of diameter were obtained, it was evaluated the cellular adhesion and a first-order kinetics was adjusted with a constant k = 0.007 min^{-1}. Celullar adhesion was observed on the microcarriers using confocal microscopy and fluorescence microscopy. Fibroblasts grown in the reactor showed that it is possible to reduce the doubling time from 23.4 until 16.3 h.**

Keywords- **microcarriers, alginate, cellular adhesion, fibroblasts, fibrin.**

I. Introduction

Cellular adhesion is the first contact that occurs between the cell and a surface or other adjacent cell [1,2]. The main cellular receptors involved in adhesion between cell and surface are integrins, which involve joints between the extracellular matrix and cytoskeletal structures and also allow the transmission of mechanical and biochemical signals involved in cell-matrix adhesion [3]. The process of cell-surface adhesion is carried out generally in anchorage-dependent cells such as fibroblasts, allowing their proliferation. It is necessary develop microcarriers that offer a surface for adhesion of fibroblasts for culturing cell in spinner type bioreactor. Microcarriers are micro-spheres which they can adhere and prolife cells until the whole area available was covered. The size of microcarriers vary from 90 µm to 300 µm diameter; for their manufacturing, polymeric materials are typically used [4].

Literature reviewed shows that 1 g of microcarriers can provide a surface equivalent to 15 culture flasks of 75 cm^2, with an area from 380 cm^2/g up to 440 cm^2/g and a density between 1.02 g/ml and 1.10 g/ml. In order to generate microcarriers that may be biocompatibles and biodegradables, human blood plasma and sodium alginate were mixed and then discharged onto a chloride calcium solution, using a coaxial nozzle for generating microspheres. To ensure fibroblast adhesion to microspheres, PLL and fibrinogen were adsorbed superficially. Finally, line 3T3 fibroblasts were cultured in a spinner reactor.

II. Methodology

Microcarriers production was developed using discharge solution technique through a coaxial nozzle using air as coaxial fluid [5]. The solution is a mixture of blood plasma and sodium alginate at 1.6% w/v; the production conditions were: air pressure 5 psi, mixture flow of 126 ml / h, a distance of 20 cm from the outlet of the nozzle to the surface of the gelling bath (calcium chloride at 3% w/v). Microcarriers obtained were sieved and those whose sizes were between 150 µm and 500 µm were collected. Microcarriers were stabilized in calcium chloride solution at 1% w/v. All assays were done by duplicate. Microcarriers were coated with Poly-L-lysine (PLL) at 0.1% w/v in isotonic saline solution (NaCl 0.9% w/v in deionized water) at 37 ° C, the content of free protein in the medium was determined following the protocol of Bradford every 10 minutes during 60 minutes[6,7]. Once the microcarriers were coated with PLL they were placed in a concentration of 35,000 microcarriers/ml and 1 mL of plasma cryoprecipitate solution (dilution 1:1000). The plate was placed on an orbital shaker (Scientific MaxQ Themo 4000) at 37 ° C and 100 rpm. The protein content was determined in the medium following the protocol of Bradford every 30 minutes during 120 minutes [7]. Finally, microcarriers were crosslinked using thrombin solution (test thrombine 30 IU / ml, Behringwerke, Marburg, Germany) at 1 IU/ml, incubated for 15 minutes at 37°C. Finally, they were stored in a saline solution supplemented with 0.5% tranexamic acid [8].

Murine fibroblasts from the 3T3 line were used for cell culture in a spinner reactor. The culture conditions employed

A. Braidot and A. Hadad (eds.), *VI Latin American Congress on Biomedical Engineering CLAIB 2014, Paraná, Argentina 29, 30 & 31 October 2014,* IFMBE Proceedings 49, DOI: 10.1007/978-3-319-13117-7_45

were: culture medium DMEM-F12, 10% inactivated fetal bovine serum, 1% antibiotic / antimycotic, temperature 37 ° C. For evaluation of cellular adhesion, 35,000 microcarriers/ml were placed in a well of plate of micro technique and trypsinized fibroblasts were added at a ratio of 10:1 (cells: microcarrier) and the cellular concentration was determined for trypan blue staining and counted in a Neubauer chamber. Finally, fibroblasts were cultured in a spinner reactor (total volume: 100 ml, medium volume: 30 ml) maintaining a concentration of microcarriers between 10,000 microcarriers and 20,000 microcarriers/ml, using an initial ratio 10:1 (cells:microcarriers). The reactor was stirred at 50 rpm for 1 minute every 90 min. Samples were taken every 12 hours in order to determine cell viability and proliferation by trypsinization and counting in a Neubauer chamber. The culture was incubated for 4-5 days.

A sample of microcarriers was fixed using 1% glutaraldehyde solution in PBSA, dehydrated using ethanol series and dried using a critical point dryer. Microcarriers were observed using scanning electronic microscopy (SEM) operated at 20 kV and atomic force microscopy (AFM) with a scan rate of 0.5Hz. For cell visualization on microcarriers, fluorescence microscopy and confocal microscopy were used with a fluorochrome CellTrace CFSE kit; cells were labeling according to protocol from CFSE kit.

III. Results and Discusions

For production of microspheres, medium viscosity alginate was used at a concentration of 1.6% w/v, according to [9]. Once the drop got in touch with calcium chloride solution, gelation occurred instantly. The Figure 1a corresponds to the surface of the spheres obtained with SEM; the surface of microcarrier was non-porous and did not offer binding proteins for cell adhesion. In addition, the cells have the same negatively charge as microcarriers and this cause an electrostatic repulsion that prevents the cell adhesion. In order to achieve cell adhesion, micro spheres were coated with PLL. This is a polycation that charges positively the surface. Also, the surface of microcarriers was modified by adsorption of PLL, such as it is shown in Figure 1b, where the presence of the protein is observed as small aggregates adhered to the alginate. The adsorption of the PLL was facilitated because this protein is a polycation, while the surface is negatively charged due to alginate charge. Once the PLL was deposited on the microcarrier, the surface has a high content of NH_3^+ groups on which could give cell adhesion. On the surface of the charged microcarriers with PLL, fibrinogen was adsorbed and then thrombin was added for crosslinking in order to guarantee the formation of fibrin networks, which can be seen in the detail of Figure 1c and 1d. The adsorption of fibrin allows cell adhesion over the surface of microcarrier due to presence of RGD sequences in this protein that promote the interaction between cellular integrins and fibrin. As can be seen in Figure 2, the changes were evident on the surface of the microcarriers as the coatings which were made with proteins.

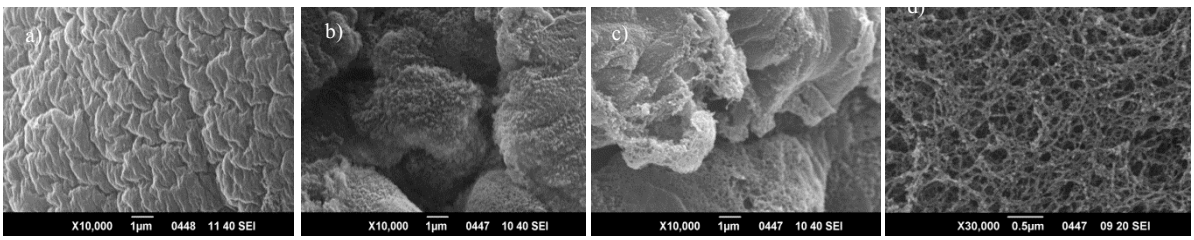

Figure. 1 scanning electronic microscopy (SEM) to microcarriers: a) Stabilized in saline solution, b) With PLL adsorbed on the surface, c) with surface adsorbed fibrinogen, d) detail of fibrin on the surface of the microcarriers.

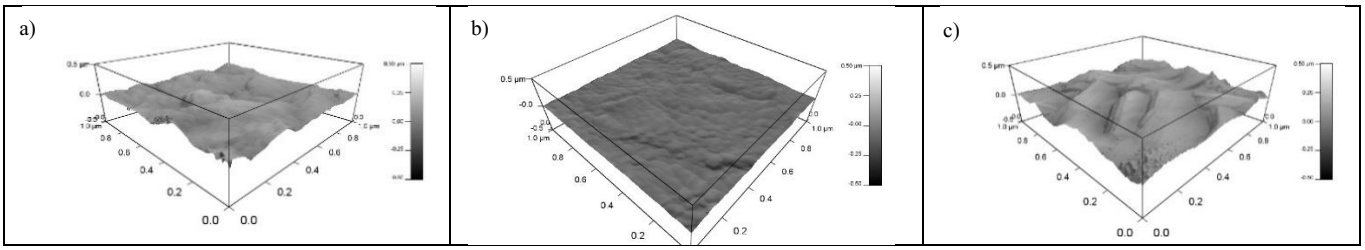

Figure.2 atomic force microscopy (AFM) of the surface of microcarriers: a.) Stabilized in saline solution, b) loaded with PLL, c) charged with fibrinogen

Amount of protein adsorbed by the microcarriers was determined over time. Adsorption kinetics is presented in Figure 3. The PLL adsorption time was 3 to 5 min. The concentration of the solution added to the wells PLL without microcarriers (blank test) remained constant over time with a value of 0.1759 ± 0.0027 mg/ml. In contrast, the free protein concentration to the well with microcarriers was 0.0479 ±0.0042 mg/ml, indicating that adsorption was presented on the microcarriers. With the known dimensions of the microspheres used (diameter 219 µm), the protein adsorbed per unit area was calculated and it was about 0.0038 mg protein/cm^2.

On the other hand, adsorption of fibrinogen onto the surface of microcarrier charged with PLL is showed in Figure 4. The maximum amount of protein adsorbed to the microcarriers in either case was reached after 60 min. Then, the residual concentration protein remained constant in the medium. In the end, the amount of protein adsorbed to the microcarriers was 2.235x10^{-4} mg protein/cm^2.

The assay of cell adhesion showed that the number of free cells in the culture medium related to the initial cell number decreases as time passes, which is associated with the cellular adhesion to the microcarriers. We adjusted a first order kinetic obtaining a time constant of 0.007 min^{-1}. In Figure 5, cells attached to the microcarrier surface can be observed. This attachment implies that the cell can bind to specific chemical groups on the surface of microcarrier (RGD sequence) using the glycoproteins present in the membrane [10].

The bioreactor assay showed that generous agitation transferred by the magnetic system prevented cells detaching from surface of the microcarrier. The use of a low concentration of the microcarriers allowed the diffusion of nutrients throughout the culture, which led to the cells to cover a wide range of the available area. Additionally, the doubling time of fibroblasts decreased from 23.40h (cultured in a plate) until 16.23h (cultured in spinner reactor). By use of fluorescence staining, it was possible to conclude the adhered cells are viable and capable to cover most of the surface of the carrier (Figure 5).

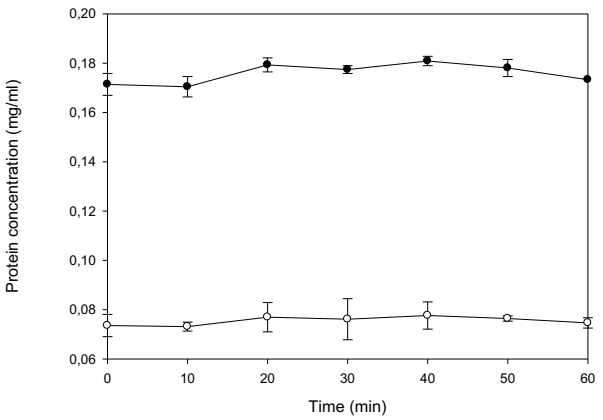

Figure 3. Protein concentration over time in the adsorption of poly-L-lysine on microcarriers plasma / alginate. ○: Wells with microcarriers, ·: Wells without microcarriers.

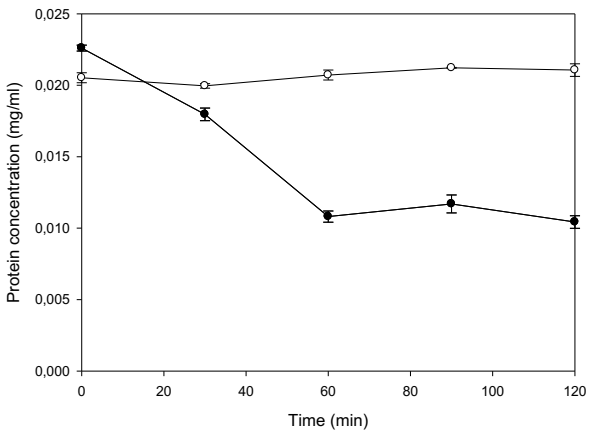

Figure 4. Protein concentration over time in the adsorption of fibrinogen plasma microcarrier / alginate coated with poly-L-lysine. ○: Wells without microcarriers, ●: Wells with microcarriers

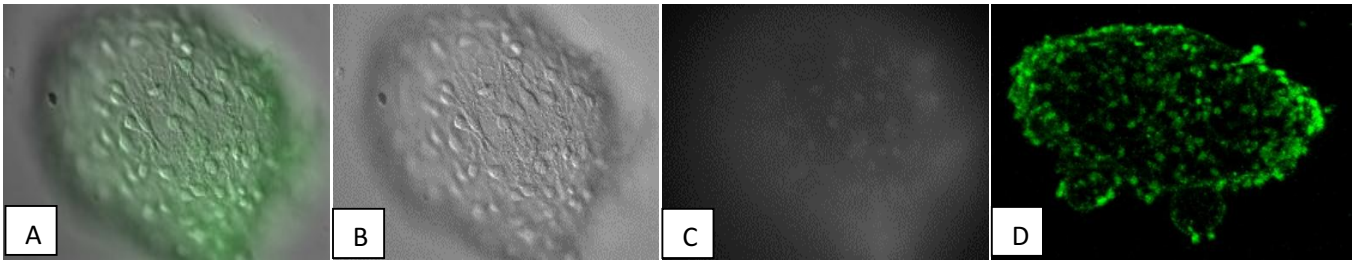

Figure 5 A-C) Fluorescence Microscopy line 3T3 fibroblasts on microcarriers on day 5 of culture reactor *Spinner.* D) Confocal microscopy of 3T3 fibroblast line on microcarriers on day 5 of culture reactor *Spinner.*

IV Conclusions

In this paper reports a new method for production of microcarriers using human blood plasma mixed with alginate. For cell adhesion improving, it was necessary the adsorption of PLL and fibrinogen on the microcarrier surface. Assays using fibroblast murine of 3T3 line proved that the surface of microcarriers allowed cell adhesion due to presence of fibrin. Furthermore, the culture was made in a spinner reactor decreasing the duplication time compared to their growing in plate. Finally, the integration of bioreactors in the proliferation of fibroblast is an alternative to static culture, and represents an innovative option in tissue engineering research.

Conflict of interest

The authors declare that they have no conflict of interest.

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