Biofunctionalization of microcarriers for the expansion of human neural stem cells

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Abstract The need to provide an unlimited supply of neural stem cells (NSCs) for scientific research and clinical applications is the drive for the development of efficient large scale bioprocesses. Microcarriers, which provide a surface for cell adhesion in suspension, combined with bioreactors are a possible scalable strategy. There are several microcarriers commercially available, however, there is a great need to design microcarriers tailored for the expansion of specific stem cell lines. This work focused on the functionalization of microcarriers' surface to enhance attachment and proliferation of long- term neural stem cell (lt-NES), when cultured under dynamic conditions. A screening of microcarriers improved by surface conjugation using two different techniques, laminin covalently bond through a 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysuccinimide (NHS) based reaction or through passive adsorption, was performed in static conditions. On the basis of those results, poly-ornithine/laminin adsorbed Plastic and laminin crosslinked Carboxyl microcarriers were compared in spinner flask culture. Carboxyl microcarriers supported lt-NES proliferation in static conditions but cell detachment was observed in spinner flask. Plastic microcarriers revealed to be more efficient in lt-NES culture in a spinner flask, reaching a maximum fold increase of 1.69. Moreover, maintenance of lt-NES phenotypic properties expanded in the latter approach was verified by expression of neural markers Nestin and Sox2. The results obtained may be the basis for future optimizations of culture parameters for lt-NES expansion.

Keywords: Microcarriers; Neural Stem Cells; EDC-sulfo NHS reaction; Adsorption

Introduction

Nowadays, importance of stem cells is widely recognized both in the scientific research field and in clinical practice. Given their unique features, the capacity to self-renew and to differentiate into other cell types, scientists envisioned stem cell as potential medical therapies, for example, to replace damaged cells or regenerative tissues or organs [1]. Neural stem cells (NSCs), in particular, are expected to be used in the future in the treatment of major neurodegenerative disorders, including, Parkinson´s and Alzheimer's diseases [2]. In order to match that expectation, large-scale technologies are required to support expansion of relevant cell numbers without compromising their functional properties. NSCs can be cultured as anchorage dependent cells, using planar surfaces like tissue culture plates or flasks [3]. Those, however aren´t suitable to generate the quantities intended for therapies.

Microcarriers provide a surface for cell adhesion while maintained in suspension. Combining microcarriers with bioreactors allows the development of scalable systems, highly controlled regarding critical culture parameters, including shear stress, temperature, pH, diffusion of nutrients, oxygen and metabolic products [4]. There are several microcarriers commercially available but there is a great need to design microcarriers tailored for the expansion of specific stem cell lines. Surface properties of the microcarriers, or of any scaffold, are known to influence cell growth and fate [5], which explains much of the work described in the literature regarding microcarrier functionalization in an attempt to mimic the *in vivo* stem cell microenvironment. Usually, surfaces for cell adhesion are coated with ECM proteins through physical adsorption [6, 7] which does not provide strong interactive forces between the cells and the substrate. It was hypothesized that such forces wouldn´t be stable in stirring conditions, resulting in the release of the ECM molecules and subsequently, the cells from the microcarriers [8]. Therefore, it was proposed that surface conjugation of microcarriers based on a covalent bond with ECM proteins would improve the stability of the protein coating and likewise the efficiency of cell attachment and proliferation.

This project aimed at the development of improved microcarriers for NSC culture. Selected microcarrier particles were thus functionalized by surface conjugation using two different techniques and a comparison of their performance for NSC culture under static and dynamic conditions was performed.

Materials and Methods

Microcarriers preparation and biofunctionalization

Different commercially-available animal-free microcarriers were tested under static conditions with different coatings: Carboxy Polystyrene (RAPP Polymere), Cytodex 1 (GE Healthcare) and Plastic (Pall Life Sciences). Carboxy Polystyrene present –COOH groups on its surface. Cytodex 1 was prepared prior to use according to manufacturer´s recommendations. All of the microcarriers were sterilized with ethanol 70% for 1h at room temperature, using a rocking platform mixer, washed with sterile PBS and kept in sterile PBS, at room temperature until functionalization and/or cell culture. The microcarriers were coated using two strategies: via a covalent bond through 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxy-Sulfosuccinimide (sulfo-NHS) reaction or through adsorption of poly-ornithine and laminin to the beads. Unless mentioned otherwise, laminin was crosslinked at a concentration of 14µg/cm^2 and adsorbed at 3µg/cm^2 .

Surface functionalization via EDCsulfo-/NHS crosslinking

Microcarriers with functional groups on the surface were coated with laminin through EDC/sulfo-NHS crosslinking. Carboxyl microcarriers were submitted to the following protocol: 30mg of beads (≈11cm^2) were prepared in 3mL activation buffer (0.1M MES (2-[morpholino]ethanesulfonic acid, Sigma)), 0.5 M NaCl at pH 6.0. Sulfo-NHS (N-hydroxysuccinimide, Acros Organics) and EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide, Sigma) were added to the microcarriers at a final concentration of 5mM and 2mM, respectively, and mixed for 30mins at room temperature. Then, excess liquid was removed and beads were washed twice with PBS and incubated overnight with the laminin (Sigma**)** solution in PBS in the desired concentration, at pH 7.2-7.5, with magnetic stirring. After overnight incubation, beads were washed twice with PBS, incubated in a 100mg/mL glycine (Sigma) solution for 1hour, at room temperature with agitation, and washed once with PBS. Quantification of the EDC/sulfo-NHS crosslinking reaction was performed through the Bradford assay. Briefly, samples of the supernatant from microcarrier crosslinking reaction with laminin solution were taken immediately after addition of the laminin solution and after overnight incubation, mixed with the dye and its absorbance measured at 595nm using a microplate reader (Infinite M200 Pro, Tecan).

Surface functionalization through adsorption

A poly-ornithine/laminin coating was performed on Cytodex 1 and Plastic microcarriers. Poly-ornithine was added for 1 hour in a rocking platform mixer. Beads were washed with sterile PBS and incubated with a 25µg/mL laminin solution in PBS for 3 hours with agitation. After that period, solution was removed and microcarriers were washed and maintained in culture media until cell seeding.

Lt-NES cell culture

The human ESC -derived lt-NES cell lines used in this (line I3; passages P43-P49) were provided by the laboratory of Professor Oliver Brüstle (Institute of Reconstructive Neurobiology, Life and Brain Center, University of Bonn, Germany). Lt-NES were cultured onto poly-ornithine/laminin (SIGMA) coated 6well-plates or T25 Flasks, in a serum-free medium which consisted in DMEM/F12 (1:1) + Glutamax (Life Technologies) containing N2 supplement (1:100; Life Technologies), 1.6g/L additional glucose (Sigma), 0.2% additional human insulin (25mg/mL, Sigma), 100Umg/mL penicillin, 100µg/mL streptomycin (Life Technologies) and supplemented with 20ng/mL human EGF, 20ng/mL FGF-2 (both from Peprotech) and B27 (1:1000; Life Technologies. Cultures were maintained at 37°C under a 5% CO_2 humidified atmosphere and medium was replaced every two days. Cells were passaged when confluent using trypsin-EDTA and counted.

Lt-NES cell expansion on microcarriers under static conditions

Microcarriers were equilibrated in culture medium at 37°C before cell seeding. For each type of microcarrier an approximate area of 3cm^2 and an initial cell density of 5×10^4 cells/cm^2 were considered. A total number of 2×10^5 (or 6×10^5) cells were seeded onto the microcarriers in 24-well (or 6-well) ultra-low attachment plates (Corning) at 37°C. Culture medium was replaced every 2 days. Cell attachment and proliferation were assessed daily in the microscope. For each condition in 24-well ultra-low attachment plate, triplicates were analyzed. After 6 days, Lt-NES were detached from the beads with trypsin-EDTA

protocol and filtrated using a cell strainer (Corning), in order to remove microcarriers. After centrifugation (1000rpm for 3min), cells were ressuspended in culture media supplemented with hEGF, FGF-2 and B27 and counted in a hemocytometer after trypan blue staining. To determine the maintenance of cell viability and morphology after microcarrier culture, cells were replated in poly-ornithine/laminin coated 24-well well-plates.

Lt-NES cell expansion on microcarriers in spinner flasks

Cell culture under dynamic conditions was performed in spinner flasks with workings volumes of 30mL (StemSpan, Stem Cell Technologies). The spinner flask is equipped with an impeller with paddles and a magnetic stirring bar and was placed over a magnetic stirring plate inside a CO_2 incubator, at 37°C. Cells were cultured on Plastic and -COOH beads prepared as previously mentioned, both functionalized with 3µg/cm^2 laminin. Cells were previously expanded in static conditions until confluence and inoculated as single cells at a final concentration of 9.4 x10^4 cells/cm^2, in a total of 10x10^6 cells. The microcarriers' equivalent area used was 108cm^2 and 107 cm^2 for Plastic and COOH beads, respectively. Cells mixed with microcarriers were incubated in a 6-well ultra-low attachment plate for 1.5 hours at 37°C in a CO_2 incubator, in approximately one third of the final volume of medium. Then, cells mixed with microcarriers were transferred to spinner flasks and incubated overnight with no agitation. The next day, continuous stirring was set to 25rpm and the final volume (30mL) was completed. After this period, continuous stirring was kept at 50rpm. Every day, half of the medium was replaced and growth factors (10ng/mL of FGF-2 and hEGF and B27 (1:1000)) were added. A static expansion control was performed to confirm the initial cell viability. Duplicate samples of 500µL of the culture were collected from the spinner flasks on a daily basis. Samples were washed with PBS and cells were detached from the beads following the trypsin passaging protocol. Viable cells were determined by counting in a hemocytometer using the trypan blue exclusion method under an optical microscope. Fold increase in cell number was calculated as the ratio between the cell number at the end of culture and the initial inoculated cell number.

Metabolite analysis

Samples were collected from the spinner flask culture and microcarriers were allowed to settle. Supernatant was centrifuged at 1500rpm for 10mins and kept at -20°C for subsequent analysis. An automatic analyzer (YSI 7100MBS, Yellow Springs Instruments) was used to determine the glucose and lactate concentrations present on the culture medium collected throughout the culture. Specific lactate production rate (q_{lac}) and specific glucose consumption rate (q_{gluc}) were calculated according to equation 1:

$$q = \frac{\Delta C}{\bar{X}\Delta t} \qquad (1)$$

Where ΔC is the lactate produced/ glucose consumed during the time period; Δt and $\bar{X}$ is the average number of cells in culture during the same period. The apparent lactate from glucose ($Y_{lac/gluc}$) yield was calculated as the ratio between q_{lac} and q_{gluc}.

Immunocytochemistry

Cells on culture plates or on microcarriers were fixed with 4% paraformaldehyde (PFA) solution (Sigma) for 10 mins. at room temperature and washed with PBS to remove the fixation agent. Next, cells were permeabilized with blocking solution 10% FCS (Life Technologies), 0,1% Triton X-100 (Sigma) in PBS for 30-60minutes at room temperature. Blocking solution was aspirated and cells were incubated overnight at 4°C with the following primary antibodies diluted in staining solution (5% FCS, 0,1% Triton X-100 in PBS): anti-Nestin (dilution 1:200, R&D Systems), anti-Sox2 (dilution1:500, R&D Systems), anti-βIII-Tubulin (dilution1:4000, Covance). After the incubation period, cells were washed with PBS and incubated with the appropriate secondary antibody conjugated to Alexa fluorophores (Alexa 546, Life Technologies) for 1h at room temperature, protected from light. The secondary antibodies used After washing with PBS, nuclear DNA was stained with DAPI (Sigma), dilution 1:10000, for 2 minutes at room temperature. Cells were washed and analyzed under a fluorescence microscope (Leica DMI 3000B).

Results and Discussion

Validation of the EDC/sulfo-NHS crosslinking in surface conjugation

In order to validate the occurrence of the covalent coupling reaction and quantify the amount of protein attached to the surface of the beads the Bradford colorimetric method was used. Supernatants of laminin-adsorbed microcarriers were also read in order to compare efficiencies of both coupling procedures**.** In *Figure 1* it is observed a decrease, although slight, in

the absorbance of the condition of the EDC/NHS reaction. A small reduction is also observed for the adsorption condition. No difference in the absorbance was observed when no protein was present in the supernatant (blank). Using the laminin standard curve (not shown), the concentration of laminin in the supernatant samples was estimated and the protein attachment efficiency of both coupling procedures was calculated based on the amount of protein which attached to the surface.

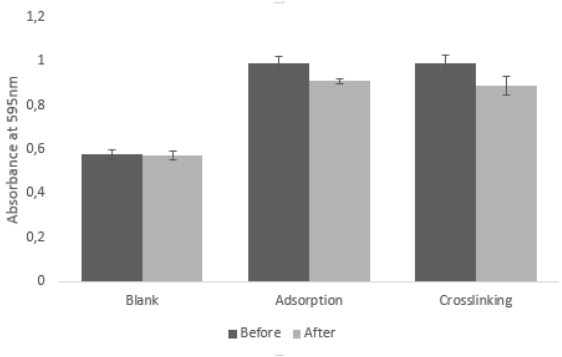

Figure 1: Absorbance of supernatant samples of laminin crosslinked beads, measured right before and after the incubation period of the reaction. Absorbance of uncoated beads (blank) and laminin adsorbed beads were measured at the same time periods. Results of crosslinking are the average of 3 independent experiments, uncoated and adsorption are the average of 2 independent experiments. Error bars represent SEM.

Covalent bonding showed to be slightly better in protein immobilization, having a protein attachment efficiency of 45% against the 41% achieved by the adsorption. The small differences in the absorbance values of the "before" and "after" readings and the apparent low efficiencies of the reactions may be due to the low specificity of the Bradford dye to laminin. Therefore, a more accurate and specific method would be needed to validate the crosslinking reaction and quantify the amount of laminin available at the surface of the beads.

Lt-NES cell culture under static conditions

A first screening of different commercially available microcarriers was performed in static conditions with different surface functionalization techniques and coatings. Plastic microcarriers coated only with poly-ornithine showed no significant improvement in cell growth compared to the uncoated conditions (*Figure 2*). On the other hand, poly-ornithine/laminin coated Plastic microcarriers provided the best result in terms of cell expansion, with visible spreading of cells on the microcarriers´ surface and cell "bridging" between

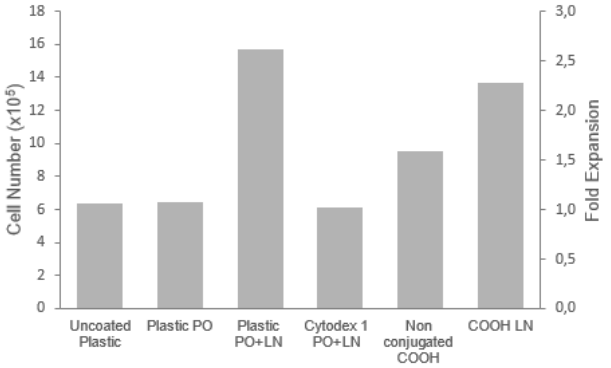

Figure 2: Cell number and fold expansion after 6 days of culture in Plastic, Cytodex 1 and –COOH beads under static conditions. PO: poly-ornithine; LN: laminin. Results of one experiment.

beads (pictures not shown). Cell expansion was also higher than observed with Cytodex 1, even with the same coating (*Figure 2)*.

Positively charged surfaces are known to promote rapid cell adhesion but are often not sufficient to sustain cell growth given the nonspecific physical interactions between the surfaces and cells [9], which may explain the poor results of poly-ornithine coated Plastic beads. Another possible explanation could be related with excessive electrostatic charge, which could be inhibiting cell spreading and growth, explaining also the poor results of Cytodex 1, since the microcarrier is naturally positive given the charged groups and was additionally coated with poly-ornithine [10]. The results obtained with poly-ornithine Plastic suggest that a poly-ornithine coating is not enough to support the attachment of lt-NES, leading to the formation of cell aggregates in suspension (pictures not shown), and thus has not a beneficial effect in relation to the uncoated beads. In contrast, poly-ornithine/laminin coated Plastic beads promoted good cell attachment to the beads surface, with few free clumps of cells in suspension (pictures not shown), probably due to the combination of the electrostatic interactions promoted by the positively charged poly-ornithine and the signaling pathways for attachment triggered by the ECM adhesion protein laminin. These surface features are more appropriate for cell spreading and cell growth, which explains the better results. Carboxyl beads conjugated with laminin exhibited a higher cell-fold expansion compared with the non-conjugated beads. Therefore, surface conjugation of –COOH beads by EDC/sulfo-NHS was effective in the enhancement of cell attachment and growth. Lt-NES from poly-ornithine/laminin adsorbed plastic and Cytodex 1 and –COOH conjugated beads maintained their neural rosette morphology after re-

plating and exhibited expression of Nestin and Sox2 (results not shown). Given these results, it was concluded that Plastic microcarriers coated with poly-ornithine/laminin and –COOH microcarriers with laminin covalently bound support lt-NES attachment and growth, with maintenance of NSC identity after re-plating. Moreover, the results depict the versatility of lt-NES to be cultured in microcarriers made of different materials.

Comparison of adsorbed and crosslinked microcarriers

Prior to spinner flask culture, different concentrations of laminin were tested to determine the optimal coating conditions, either by adsorption on Cytodex 1 and Plastic beads, or EDC/NHS crosslinking in Carboxyl microcarriers. The highest cells numbers were obtained with the covalent bounded beads, all of them being superior to their adsorbed counterparts (*Figure 3*). Apparently, there is no proportional relation between the amount of laminin coated and the number of cells. Aggregation of beads and "cell bridging", probably due to the absence of agitation, were observed in all of the conditions, with the exception of all of laminin crosslinked carboxyl beads experiments, after a few days of culture (pictures not shown).
Although the 2µg/cm^2 laminin crosslinked –COOH beads gave slightly better results regarding cell number, the same concentration of the protein was shown to be suboptimal for the adsorption condition. It is important to note, though, that the experiment was performed only once and the difference may be within the experimental error. However, to compare the performance of the adsorbed and crosslinked beads in spinner flask cultures, it was decided to choose an identical laminin concentration for both methods. Given the previous results, poly-ornithine coated Plastic microcarriers and –COOH microcarriers, both conjugated with 3µg/cm^2 of laminin, were chosen to be compared for the culture of lt-NES cells in spinner flasks, since this concentration was one of the best for both methods.

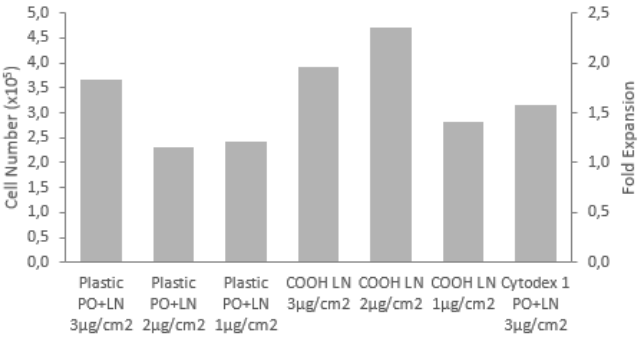

Figure 3: Cell numbers after 6 days of culture in adsorbed Plastic and Cytodex 1 beads and conjugated -COOH beads, with different laminin concentrations. PO: poly-ornithine

Lt-NES cell culture under dynamic conditions

Envisaging the development of a large-scale bioprocess for lt-NES cell production, a preliminary experiment in dynamic culture conditions was performed, using spinner flasks as model bioreactors. An initial concentration of 10g/L of microcarriers, which corresponds approximately to an equivalent surface area of 108cm^2, was used, and 10x10^6 cells were seeded. A concentration of 3µg/cm^2 of laminin was used to coat the microcarriers. Agitation was only applied after 24h to promote an adequate environment for the cells to attach the beads.

Carboxyl microcarrier culture in spinner flask

Lt-NES culture in carboxyl microcarriers in a spinner flask was only kept for five days since cell survival reduced throughout the culture period and no fold increase in cell number was registered (*Figure 4*). Agitation was started in day 1 and increased in day two, being the lowest point of the culture registered the next day, which indicates optimum values for agitation speed weren´t applied. Adhesion of cells onto the beads and overall homogeneous occupancy was visible in days one and two, but extensive detachment followed afterwards with cell clumps being observed in the medium (*Figure 5*). It must be pointed out that, although the microcarrier weight used was the same for both spinners, it was clear a reduction in the quantity of the –-COOH microcarriers after the laminin immobilization steps. This reduction could be due to loss of particles explained by the high number of washing steps that those had to be subjected to, given the EDC/NHS reaction protocol.

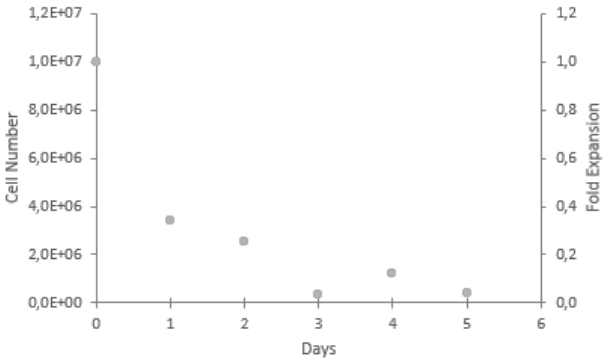

Figure 4: Growth curve of lt-NES cultured in spinner flasks with 3µg/cm^2 laminin crosslinked –COOH beads. Results of one experiment.

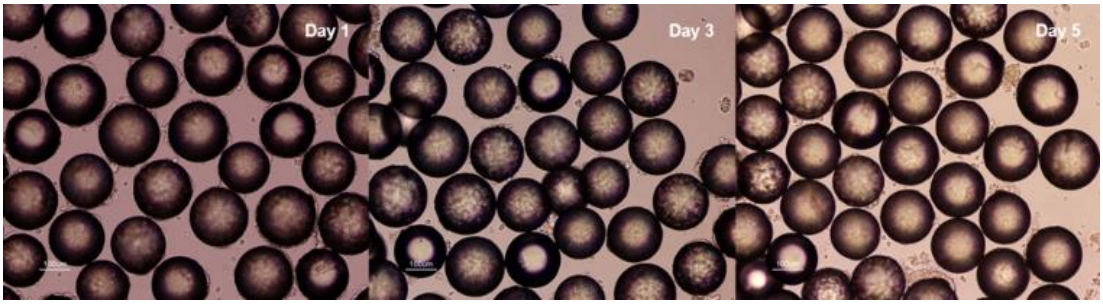

Figure 5: lt-NES cell occupancy on –COOH beads harvested in day 1, 3 and day 5 of culture in spinner flask.

Unsuccessful scale up using this same system is reported in the literature: despite –COOH conjugated beads supported hPSCs growth in static cultures, extensive detachment was observed under stirring conditions [8]. It is hypothesized that adhesion of cells in this type of system is not strong enough to prevent detachment under stirring. As future work, additional conjugation of the –COOH beads with pLL adsorption, a synthetic cationic polymer that enhances cell attachment and promotes the adsorption of ECM proteins, could circumvent this problem, as it is described as well by Fan *et al [8]*. The combination of all of the previous factors may explain the failure of laminin crosslinked carboxyl beads in lt-NES culture under dynamic conditions. A less amount of beads resulted in less surface available for adhesion, that together with the weak cell adhesion forces in an agitated environment led to detachment and poor survival.

Levels of glucose and lactate were evaluated throughout spinner flask culture (*Figure 6*). No significant glucose consumption rates were observed, which is consistent with the low cell number and cell death, being only noticeable a slight reduction in concentration in the first days of culture when a higher number of cells was counted. The lactate concentrations registered were below the values reported as inhibitory (35.4mM for human mesenchymal stem cells) [11], reaching a maximum value of 9.16mM, thus, theoretically, lactate accumulation didn´t had toxic effects in the system. A reduction of lactate levels occurs from day 3 until the end of culture, corresponding to the lowest cell numbers reached in the culture. The average molar ratio of lactate produced over glucose consumption $Y_{lac/gluc}$ was 1.91, which is approximate to the maximum theoretical value of 2, correspondent to the glucose metabolism by anaerobic glycolysis.

Given the present data, it may be concluded that results obtained in static microcarrier cultures cannot be directly translated to dynamic environments.

Laminin crosslinked –COOH beads efficiently supported lt-NES cell attachment and growth in tissue culture plates, but weren´t capable to do so when cultured under dynamic conditions in a spinner flask.

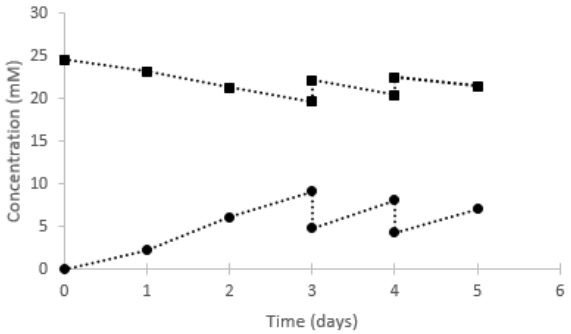

Figure 6: Concentration profile of glucose (■) and lactate (●), in mM, during lt-NES -COOH beads culture in spinner flask. Results of one experiment.

Plastic microcarrier culture in spinner flask

Simultaneously to the carboxyl beads spinner flask culture, a poly-ornithine/laminin adsorbed Plastic microcarrier spinner flask was also performed but no exponential growth phase was identified in *Figure 7*. At day 1 of culture, about 60% of the inoculated cells adhered to the plastic beads, corresponding to almost twice the value for carboxyl beads, which presented 34% of seeding efficiency. At the end of culture, the spinner flask promoted a fold expansion of 1.54 in cell number, with a maximum of 1.69 detected at day 9. In addition, adherent cells exhibited a 2.5-fold increase in cell number. A reduction in cell number was observed from day 2, probably due to poor cell adaptation to the increase in agitation speed from 25rpm to 50rpm. Despite this, microcarrier examination by microscopy revealed a homogeneous and monolayer occupancy of the beads with cells throughout culture (*Figure 8*). This observation suggested that the culture was facing a limitation in terms of surface area available for cell growth that at this point was about 108cm^2. For this

reason, additional microcarriers with a total surface area of 72cm^2, corresponding to 66% of the initial surface area available, was added by day 5. The increase registered from day 5 afterwards indicates that cells were able to repopulate the new beads.

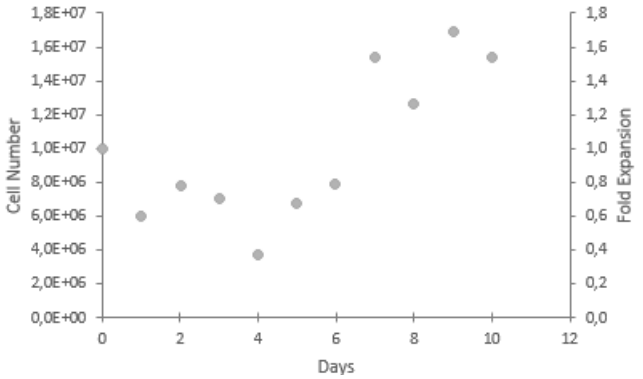

Figure 7: Growth curve of lt-NES cultured in spinner flasks with 3µg/cm^2 laminin coated plastic beads. Results of one experiment.

Cell density throughout the culture period was plotted to understand if microcarriers were confluent before the addition of the empty beads and at the end of the culture (*Figure 9)*. Cell density reduced at day 6, due to the increase in more than 50% of the available surface area, not followed by a significant increase in cell number. Probably, the number of cells inoculated was too great for the surface area available, explaining the reduced growth in the first days of culture. The new beads added in day 5 were thus rapidly populated.

The expression of neural progenitor markers Nestin, Sox2 and the neuronal differentiation marker Tuj1 was assessed by immunocytochemistry, directly on the microcarriers (*Figure 10*). Cells retained expression of Nestin and Sox2, and no detection of Tuj1 was registered. Similar expression patterns were registered for Nestin and Sox2, for cells harvested from the microcarriers, while it was detected residual

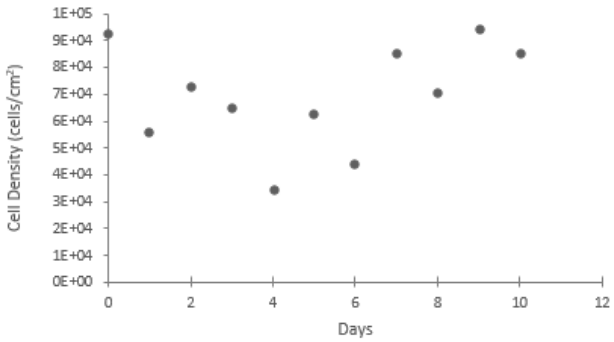

Figure 9: Cell density over time (days). Initial surface area available was 108 cm^2, increased in day 5 to 180 cm^2. Results of one experiment.

expression of Tuj1 (*Figure 11)*. Taken together, the immunocytochemistry results indicate that lt-NES can be propagated on poly-ornithine/laminin coated plastic microcarriers in a stirred environment without loss of multipotency marker expression. Confirmation of the expression of neural progenitor markers by RT-PCR would also be necessary, as well as determination of the differentiation potential of lt-NES, after culture in poly-ornithine Plastic beads in a spinner flask.

The concentration profiles of glucose and lactate throughout culture period were plotted. In *Figure 12*, no complete depletion of glucose is observed and the

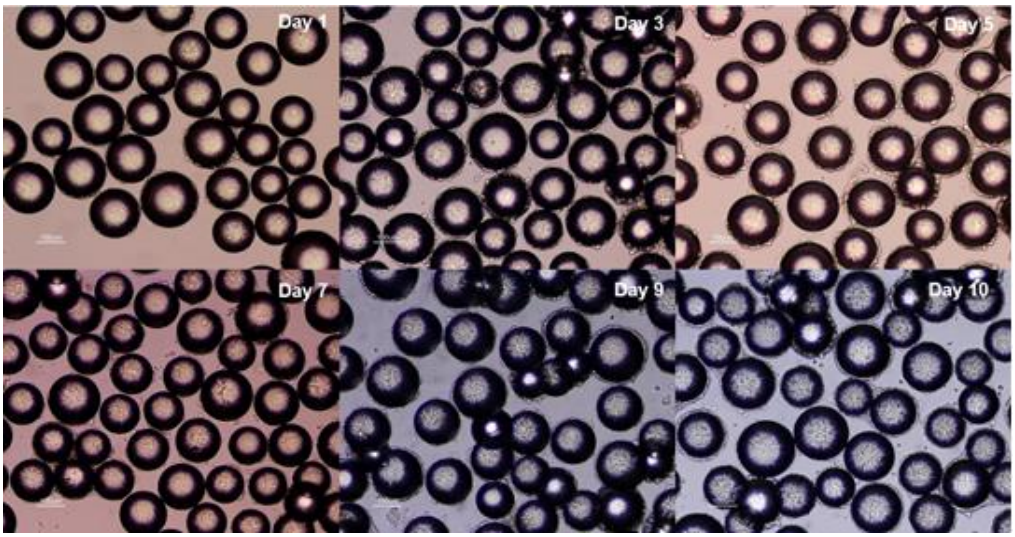

Figure 8: lt-NES cell occupancy on Plastic beads harvested in days 1, 3, 5, 7, 9 and 10 of culture in spinner flask.

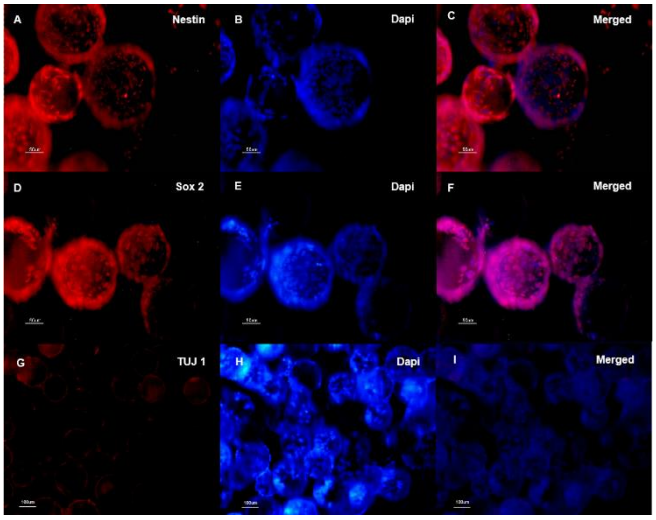

Figure 10: Immunostaining of cells for NSC markers Nestin (A-C), Sox 2 (D-F) and neuronal differentiation marker Tuj1 (G-I) expression in plastic beads cultured in spinner flasks.

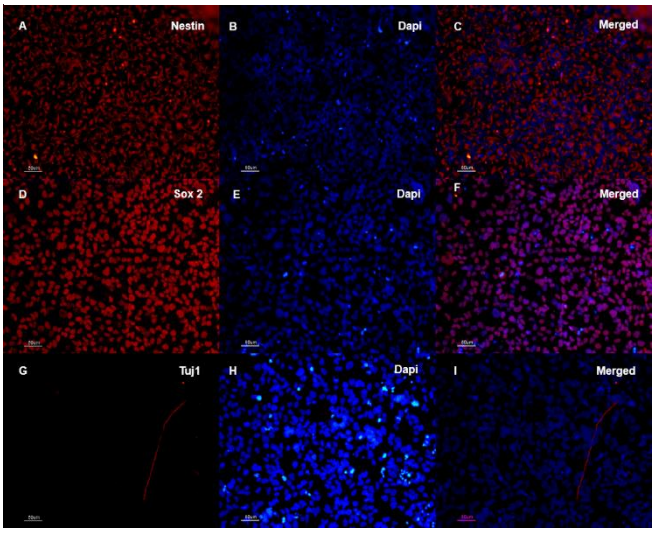

Figure 11: Evaluation of NSC markers Nestin (A-C), Sox 2 (D-F) and neuronal differentiation marker Tuj1(G-I) expression in cells harvested from plastic beads cultured in spinner flasks and replated in poly-ornithine/laminin tissue

lower concentration detected is 14.65mM. Therefore, glucose availability wasn´t a limiting factor for cell growth. A decrease in glucose concentration occurs until day three and slightly from day 6 until the last time point. Lactate concentrations, on the other hand, increased during the entire period, with exception of day 6. Lactate reached a maximum concentration of 17mM, which is below the growth-inhibitory concentration value for human mesenchymal stem cells. Specific lactate production (q_{lac}) and glucose consumption (q_{glu}) rates were calculated before and after the addition of new microcarriers to evaluate possible differences in cell metabolism which could explain the results (*Table 1*). A higher glucose consumption rate as well as lactate production rate were obtained in the first stage of the culture. After the addition of beads, the average molar ratio of lactate produced over consumed glucose increased to 2mM, which indicates a less efficient glucose metabolism. The overall average molar ratio of lactate produced over consumed glucose $Y_{lac/gluc}$ was determined to be 1.69. The higher concentration of lactate in the later stages of culture led to a decrease in medium pH which in turn caused medium color transition to yellow. This is known to be detrimental for cell proliferation and may explain the lack of an exponential growth phase.

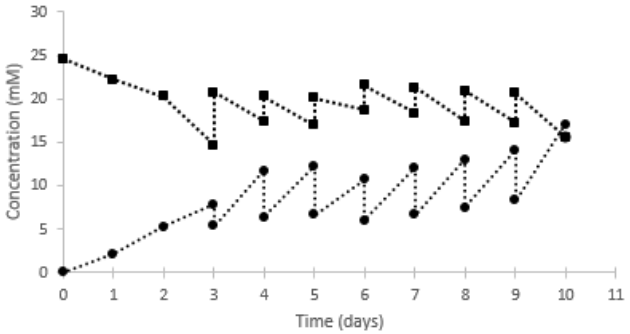

Figure 12: Concentration profile of glucose (■) and lactate (●), in mM, during lt-NES plastic beads culture in spinner flask. Results of one experiment.

Table 1: Average specific glucose consumption (q_{glu}), specific lactate production (q_{lac}) and average molar ratio of lactate produced over glucose consumption in different time periods of Plastic microcarrier in spinner flask culture.

Time period (days)	0-5	6-10	0-10
Average q_{glu} [mmol/(cell.day)]	5.17E-07	2.59E-07	3.88E-07
Average q_{lac} [mmol/(cell.day)]	6.78E-07	5.05E-07	5.91E-07
$Y_{lac/gluc}$	1.33	2.04	1.69

Based on the previous data, Plastic beads coated with poly-ornithine/ laminin, as opposed to beads with laminin covalently bound, support lt-NES cell attachment and proliferation without visible alterations in NSC phenotype. However, the culture system tested is not efficient and future work should focus on the optimization of initial microcarrier concentration, initial cell inoculation, agitation speed and schemes, and culture free of xenogeneic products, replacing mouse laminin for human or recombinant laminin for example,

and in defined conditions, which was not the case given the use of B27. Considering cost-effective cell cultures, a profound study to determine the optimal concentration of laminin used for coating without compromising the culture performance under dynamic environments would also be valuable.

Conclusions

Functionalization of the microcarriers aims to provide surface cues capable of enhancing cell attachment and growth. For this purpose, laminin, an ECM protein, was conjugated to different beads either through a covalent reaction, EDC/sulfo-NHS, or physical adsorption. It was hypothesized that protein immobilization using the covalent link would be more resistant in a spinner flask culture when compared to passive coatings. Quantitation of the laminin immobilized using the Bradford assay was similar for both coupling procedures, but given the low specificity of the Bradford dye to the laminin, other methods must be considered to get conclusive remarks.

Screening of microcarriers in static environment showed that the identical coating conditions in different microcarriers does not result in similar cell expansion numbers. Cytodex 1, for example, did not exhibit remarkable results, despite the positive charges present throughout the matrix. Therefore, optimal coating parameters, for example laminin concentration, must be tested for each type of microcarrier in order to develop cost-effective scalable systems. It is also important to replicate the experiments tested to discard experimental errors.

It is also clear from this work that the results obtained with microcarrier cultures in static conditions are not directly translated to stirring environments. That was the case with –COOH beads, which supported expansion of lt-NES in tissue culture plates, however, they were inefficient in the retention of cells to the beads in spinner flasks. Since laminin covalent bonding did not result in an advantageous feature in dynamic conditions, it is proposed an additional pLL adsorption coating, which was already demonstrated in the literature to be successful in the cultivation of hPSCs in stirred suspensions vessels [8]. It is also of great importance to evaluate different agitation schemes and speeds to prevent cell detachment. Poly-ornithine/laminin Plastic microcarriers yielded the most promising results for the proliferation of lt-NES cells in spinner flask. After 9 days in culture, a maximum 1.69 fold increase in cell number was registered, and maintenance of the neural stem/progenitor markers, such as Nestin and Sox2, was confirmed by immunocytochemistry. Despite the reduction in cell growth in the first days of culture, homogeneous and monolayer occupancy of the microcarriers was observed, which suggests that initial microcarrier concentration or initial cell density are candidate parameters for optimization. Furthermore, this culture system was not performed in defined conditions, given the use of medium supplement B27, whose formulation is not known. Xeno-derived products must also be discarded or replaced, since are sources of variability and contamination. For instance, mouse laminin could be replaced by human laminin, however, the high costs associated to the production of laminin from such a source would hinder the scale-up of this system. In addition, further characterization of the dynamically expanded cells would be necessary to validate culture using Plastic microcarriers in stirred vessels. Additional work should include an analysis to the gene expression of specific markers by RT-PCR and to differentiation potential, by induction of differentiation into neurons and glia, to confirm phenotypic and stemness properties, respectively. Controlling lactate production to prevent acidification of the medium, by monitoring culture pH could be achieved through operation under perfusion or by adding a basic solution.

In future work, it would also be interesting to study biodegradable microcarriers in the culture of lt-NES, such as gelatin or alginate, given the ease of separation of cells from the beads while theoretically providing a more safe environment by preventing the aggressive action of enzymatic disaggregation and reducing the costs. Other types of bioreactor configurations, for example, rotary wall vessels, or with different types of impellers, may be used to minimize the shear forces exerted over the cells.

Considerable progress is still required before NSCs potential can be fully exploited in cell therapy, drug discovery or disease modeling. Native NSCs microarchitecture, interactions with neighboring cells and exposure to biochemical signals, must be extensively explored so researchers could better mimic *in vitro* the neurogenic niches, with the purpose of providing a suitable and safe environment for cell attachment and proliferation. Simultaneously, development of cost-effective and reproducible systems is of key importance for the production of the large number of cells required for biomedical applications.

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