

Evaluation of Macroporous and Microporous Carriers for CHO-K1 Cell Growth and Monoclonal Antibody Production

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The emergence of microcarrier technology has brought a renewed interest in anchorage-dependent cell culture for high-yield processes. Well-known in vaccine production, microcarrier culture also has potential for application in other fields. In this work, two types of microcarriers were evaluated for small-scale monoclonal antibody (mAb) production by CHO-K1 cells. Cultures (5 ml) of microporous Cytodex 3 and macroporous CultiSpher-S carriers were performed in vented conical tubes and subsequently scaled-up (20 ml) to shake-flasks, testing combinations of different culture conditions (cell concentration, microcarrier concentration, rocking methodology, rocking speed, and initial culture volume). Culture performance was evaluated by considering the mAb production and cell growth at the phases of initial adhesion and proliferation. The best culture performances were obtained with Cytodex 3, regarding cell proliferation (average $1.85 \pm 0.11 \times 10^6$ cells/ml against $0.60 \pm 0.08 \times 10^6$ cells/ml for CultiSpher-S), mAb production (2.04 ± 0.41 µg/ml against 0.99 ± 0.35 µg/ml for CultiSpher-S), and culture longevity (30 days against 10-15 days for CultiSpher-S), probably due to the collagen-coated dextran matrix that potentiates adhesion and prevents detachment. The culture conditions of greater influence were rocking mechanism (Cytodex 3, pulse followed by continuous) and initial cell concentration (CultiSpher-S, 4×10^5 cells/ml). Microcarriers proved to be a viable and favorable alternative to standard adherent and suspended cultures for mAb production by CHO-K1 cells, with simple operation, easy scale-up, and significantly higher levels of mAb production. However, variations of microcarrier culture performance in different vessels reiterate the need for optimization at each step of the scale-up process.

Keywords: Monoclonal antibody, microcarrier, Cytodex 3, CultiSpher-S, Chinese hamster ovary cells

Introduction

Currently, most large-scale processes using mammalian cell culture, as well as a great number of small-scale systems, rely on suspended culture methods. These suspension systems offer advantages over the common anchorage-dependent culture, by providing higher surface-to-volume ratios that result in increased levels of cell density and production [27]. However, it is not always feasible to switch

cells from their normal anchorage-dependent growth [44] to suspended culture, and the use of adherent cells is sometimes preferred for certain applications (e.g., vaccine production) [15, 17]. As a consequence, much work has been done in the field of anchorage-dependent culture to create and optimize systems that increase the surface area available for cell growth. Such systems include multilayered stacked plate systems (e.g., Cell Factory and Cell Cube) [4, 19, 48], roller bottles [10, 33], and immobilization systems/

reactors (small beads, gel particles, membranes, and fibers) [9, 11, 16, 21, 23, 24, 30, 31, 34, 39, 45, 46]. Among these, microcarrier culture, first conceived by van Wezel [35], is probably the most well-known technology for large-scale adherent culture [22, 49]. By growing adherent cells on microscopic spheres that are kept in suspension by gentle stirring [6, 7, 28, 35, 44], the best characteristics of both anchorage-dependent and suspended cultures are combined. Indeed, microcarrier culture allows the cultivation of cells at their normal adherent growth mode, without need for cell adaptation, and simultaneously increases the surface area available for cell adhesion and growth, resulting in higher levels of cell density and productivity [6, 18, 29, 32, 35].

The success of microcarrier culture is dependent on the particular characteristics of the carriers, such as size, density, and surface properties (*e.g.*, charge, adhesion properties and microscopic visibility) [8, 27], which will have a strong impact on cell adhesion and growth as well as on the ease of culture operation/handling. Although a variety of microcarriers are available, they can be generally divided into two structural types: microporous and macroporous. The microporous carriers (*e.g.*, Cytodex) have a small pore size that results in cell attachment and growth only on their external surface [8]. On the other hand, macroporous carriers (*e.g.*, Cytopore, Cytoline, and CultiSpher) have larger pores and inner channels that result in cell colonization and proliferation inside. This offers advantages over microporous carriers, such as increased surface areas and consequently higher cell densities and productivities, as well as extra protection against shear stress for the entrapped cells [1, 8, 26, 27].

Cultures of both micro- and macroporous carriers are mostly known for their application in viral vaccines production [5, 25, 43]. However, they can also be used for the cultivation of different anchorage-dependent cells [14, 36, 42] and the production of recombinant therapeutics [6, 20, 40, 41]. In the field of recombinant therapeutics, monoclonal antibodies (mAbs) are increasingly important, with large-scale production commonly relying on suspended cultures of Chinese hamster ovary (CHO) cells [2, 3, 12, 47]. The microcarrier technology can be a feasible and advantageous alternative for mAb production, but such application has not yet been adequately explored, as the lack of literature on the subject demonstrates. In this context, the purpose of the present study was to evaluate and optimize the application of microcarrier culture for the small-scale growth and mAb production of CHO cells. Cultures of these cells were performed in microporous Cytodex 3 and

macroporous CultiSpher-S carriers and compared at different culture conditions (varying initial cell concentration, microcarrier concentration, initial culture volume, rocking speed, and rocking mechanism). Culture performance was evaluated in terms of both cell growth (divided into two phases: cell adhesion and cell proliferation) and mAb production.

Materials and Methods

Cells and Cell Preparation

A CHO-K1 cell line (ATCC, U.K., CCL-61) transfected with the CAB051 monoclonal antibody (Biotecnol SA, Portugal) by OSCAR gene amplification technology (Edinburgh University, Scotland) was used in this study [13].

Prior to each experiment, cells were grown in 75 cm² flasks, in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, Spain, D6546) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Spain, F7524), and 1× hypoxanthine-aminopterin-thymidine (HAT, Sigma-Aldrich, Spain, H0262), at 37°C and a humidified atmosphere at 5% CO₂. After cultures reached 70–80% confluence, cells were detached with trypsin (Sigma-Aldrich, Spain, T4049), and the cell viability and concentration were determined by the trypan blue (Sigma-Aldrich, Spain, T8154) exclusion method, using a hemacytometer. The concentration of the cell suspension was then adjusted to the values needed for each assay.

Microcarrier Preparation

The microporous Cytodex 3 (Sigma-Aldrich, Spain, C3275) and the macroporous CultiSpher-S (Sigma-Aldrich, Spain, M9043) carriers were used in this study. Their most important features are shown in Table 1.

All the glass material that contacted the microcarriers was previously siliconized to prevent loss of microcarriers by adhesion to the surfaces. For this, a small volume of Sigmacote (Sigma-Aldrich, Spain, SL2) was spread through the glass surfaces, which were then left to air dry in a hood and finally rinsed with distilled water.

The dry microcarriers were swollen and hydrated in calcium- and magnesium-free phosphate-buffered saline (PBS, prepared with 137 mM NaCl (Sigma-Aldrich, Spain, 71383), 2.7 mM KCl (Sigma-Aldrich, Spain, P5405), 10 mM Na₂HPO₄ (Sigma-Aldrich, Spain, 71637), and 2 mM KH₂PO₄ (Sigma-Aldrich, Spain, 60239), at pH of 7.4) (100 ml/g Cytodex 3 and 50 ml/g CultiSpher-S) for at least 3 h for Cytodex 3 and 1 h for CultiSpher-S, at room temperature. The microcarriers were then sterilized by autoclaving (121°C, 15 min, 15 psi) after an additional procedure of washing with calcium- and magnesium-free PBS (50 ml/g microcarrier) for Cytodex 3. The microcarriers were then stored at 4°C. Prior to use, the sterilized microcarriers were allowed to settle, the supernatant was decanted, and the microcarriers were washed twice with culture medium (50 ml/g microcarrier) for Cytodex 3 and once

Table 1. Main characteristics of microporous Cytodex 3 and macroporous Cultispher-S carriers (data provided by manufacturers).

Feature	Cytodex 3	Cultispher-S
Material of construction	Cross-linked dextran matrix coated with a thin layer of collagen	Highly cross-linked gelatin-based matrix
Particle diameter (μm)	141-211	130-380
Average pore diameter (μm)	-	20
Density (g/ml)	1.04	1.04
Approximate number of microcarriers (/g dry weight)	3,000,000	800,000
Approximate area (cm^2/g dry weight)	2,700	15,000

with calcium- and magnesium-free PBS (50 ml/g microcarrier) and twice with culture medium (50 ml/g microcarrier) for Cultispher-S.

Culture Procedure

Cultures were initially performed in 50 ml vented conical tubes (Inopat, Portugal, 87050). The two best sets of conditions were scaled-up to 250 ml shake flasks (Sigma-Aldrich, Spain, CLS431144).

Cultures in vented conical tubes. CHO-K1 cell cultures (5 ml) in both Cytodex 3 and Cultispher-S microcarriers were performed in 50 ml vented conical tubes. The assays were divided into two parts: (i) phase of initial adhesion to the microcarriers, consisting of the first 6 h of culture, and (ii) phase of cell proliferation in the microcarriers. Different culture conditions were assessed, including

initial cell concentration, microcarrier concentration (tested only for Cultispher-S according to the manufacturer's recommendation), rocking mechanism used at the phase of initial adhesion and at the phase of cell proliferation, rocking speed (tested only for Cytodex 3 according to the manufacturer's recommendation), and initial culture volume. The assays performed and corresponding conditions are described in Table 2. For each assay, the conical tubes were inoculated with the prepared CHO-K1 cells and microcarriers (both in culture medium) at the corresponding concentrations and volume of inoculation. The tubes were then incubated at 37°C, 5% CO₂, following the rocking conditions (Sky Line Orbital Shaker DOS-20S) of each assay. To ensure identical inclination angles in all the assays, the conical tubes were placed in a support with a fixed 30° inclination. For the assays where only

Table 2. Culture conditions tested for CHO-K1 cell growth and monoclonal antibody production in 5 ml cultures of Cytodex 3 and Cultispher-S microcarriers in 50 ml vented conical tubes.

Assay	Initial volume	Initial cell concentration (cell/ml)	Microcarriers (g/l)	Rocking mechanism	Rocking speed (rpm)
CYTODEX 3					
CYT1	Total	2×10^5	3	Pulse followed by continuous ^a	60
CYT2	Total	4×10^5	3	Pulse followed by continuous ^a	60
CYT3	Total	2×10^5	3	Continuous	60
CYT4	Total	4×10^5	3	Continuous	60
CYT5	Total	2×10^5	3	Pulse followed by continuous ^a	40
CYT6	Total	2×10^5	3	Continuous	40
CYT7	Half ^b	2×10^5	3	Pulse followed by continuous ^a	60
CULTISPHER-S					
CUL1	Total	2×10^5	1	Pulse followed by continuous ^a	60
CUL2	Total	4×10^5	1	Pulse followed by continuous ^a	60
CUL3	Total	2×10^5	1	Continuous	60
CUL4	Total	4×10^5	1	Continuous	60
CUL5	Total	2×10^5	2	Pulse followed by continuous ^a	60
CUL6	Total	2×10^5	2	Continuous	60
CUL7	Half ^b	2×10^5	1	Pulse followed by continuous ^a	60

^aPulse for the phase of initial adhesion, consisting of 1 min rocking at each 30 min; Continuous for the phase of cell proliferation.

^bThe remaining volume was added at the end of the phase of initial adhesion (after the first 6 h of culture).

Table 3. Culture conditions tested for CHO-K1 cell growth and monoclonal antibody production in 20 ml cultures of Cytodex 3 and CultiSpher-S microcarriers in 250 ml shake flasks.

Assay	Initial volume	Initial cell concentration (cell/ml)	Microcarriers (g/l)	Rocking mechanism	Rocking speed (rpm)
CYTODEX 3					
CYT-S1	Total	2×10^5	3	Pulse followed by continuous ^a	60
CYT-S2	Total	4×10^5	3	Pulse followed by continuous ^a	60
CULTISPHER-S					
CUL-S1	Total	4×10^5	1	Pulse followed by continuous ^a	60
CUL-S2	Total	4×10^5	1	Continuous	60

^aPulse for the phase of initial adhesion, consisting of 1 min rocking at each 30 min; Continuous for the phase of cell proliferation.

half of the culture volume was used during the phase of initial adhesion (first 6 h of culture), the remaining volume was added after that time. During the phase of cell proliferation, the medium was changed every day (half the volume).

Cultures in shake flasks. Cultures of 20 ml of CHO-K1 cells in microcarriers were performed in 250 ml shake flasks, for the two sets of conditions that achieved the best mAb production results in the preliminary conical tube experiments (Table 3). The assays were also divided in the phases of initial cell adhesion and cell proliferation, and for each assay the shake flasks were inoculated with CHO-K1 cells and microcarriers at the corresponding concentrations, and incubated at 37°C, 5% CO₂. During the phase of cell proliferation, the culture medium was changed every day (half the volume).

Culture Monitoring

For the phase of initial adhesion, samples were taken hourly and suspended cells were counted using a hemacytometer and the trypan blue exclusion method. The concentration of suspended cells ($C_{\text{suspended cells}}$) was determined according to Eq. (1) and the concentration of adhered cells ($C_{\text{adherent cells}}$), obtained by subtracting this value from the initial cell concentration, by Eq. (2).

$$C_{\text{suspended cells}} = N_{\text{cells}} \times 10^4 \times F \quad (1)$$

where N_{cells} is the average number of cells counted in four squares of the hemacytometer, and F is the dilution factor of the sample.

$$C_{\text{adherent cells}} = C_{\text{cell inoculum}} - C_{\text{suspended cells}} \quad (2)$$

where $C_{\text{cell inoculum}}$ is the concentration of cells inoculated in the culture, and $C_{\text{suspended cells}}$ is the concentration of cells in suspension.

During the phase of cell proliferation, samples were taken daily to monitor cell growth and mAb production. MAb production was assessed by enzyme-linked immunosorbent assay (ELISA), as described below. Cell growth was monitored by cell counting, after releasing the cells from the microcarriers, by enzymatic digestion with trypsin. First, to standardize results, the number of microcarriers of each sample was counted by microscopic observation. After this, the sample was transferred to a 2 ml tube,

the microcarriers were allowed to settle, and the supernatant was removed. After a wash with PBS, a trypsin solution was added and the sample incubated for 10–15 min, with occasional stirring (after this time, CultiSpher-S microcarriers disintegrate owing to their gelatin matrix; Cytodex 3 microcarriers maintain their structure). Fresh medium was added to the microcarriers to stop the trypsin reaction, and cells were counted using a hemacytometer and the trypan blue exclusion method.

Concentration of cells in the culture ($C_{\text{cell/ml}}$) was determined according to Eq. (3).

$$C_{\text{cell/ml}} = C_{\text{cell/microcarrier}} \times C_{\text{microcarriers/ml}} \quad (3)$$

where $C_{\text{cell/microcarrier}}$ is the concentration of cells adhered per microcarrier as determined by Eq. (4), and $C_{\text{microcarriers/ml}}$ is the concentration of microcarriers in culture in number of microcarriers/ml, as obtained from Eq. (5).

$$C_{\text{cell/microcarrier}} = \frac{[(N_{\text{cell}}/4) \times 10^4] \times F \times V_{\text{sample}}}{N_{\text{microcarriers in sample}}} \quad (4)$$

where N_{cell} is the total number of cells counted in the hemacytometer, F is the dilution factor of the sample, V_{sample} is the volume of sample in milliliters, and $N_{\text{microcarriers in sample}}$ is the total number of microcarriers counted in the sample.

$$C_{\text{microcarriers/ml}} = C_{\text{microcarriers in g/ml}} \times N_{\text{microcarriers/g dry weight}} \quad (5)$$

where $C_{\text{microcarriers in g/ml}}$ is the concentration of microcarriers used in the culture in grams of dry weight per milliliter, and $N_{\text{microcarriers/g dry weight}}$ is the approximate number of microcarriers per gram of dry weight (data provided by manufacturer, shown in Table 1).

Antibody Quantification by ELISA

Daily samples taken from the culture were analyzed for mAb productivity by ELISA, following an optimized procedure described in the confidential SOP 2008-01 ANL property of Biotecnol SA, Portugal. Briefly, 96-well plates (CoStar, U.S.A., 9018) were coated with capture antibody overnight and then blocked for 45 min at room temperature. Dilutions of the samples were added, as well as a standard of known concentration and a quality control, and the plates were incubated for 2 h at 37°C. The detection antibody

(anti-human IgG (γ -chain specific)-peroxidase antibody produced in goat; Sigma-Aldrich, Spain, A6029) was then added, and the plates were incubated for 2 h at room temperature, after which a 3,3',5,5'-tetramethylbenzidine (TMB, Sigma-Aldrich, Spain, T0440) substrate solution was added and allowed to react for 10 min, at room temperature. After stopping the reaction, the absorbance was read at 450 nm, and the mAb production of each sample was determined. For this, the calibration curve of the ELISA was determined using R software (The R Foundation for Statistical Computing) to obtain the values of the four-parameter logistic Eq. (6).

$$Abs_{450} = d + \frac{a-d}{1 + \left(\frac{C_{mAb}}{c}\right)^b} \quad (6)$$

where Abs_{450} is the absorbance read at 450 nm, a is the estimated response at zero concentration, b is the slope factor, c is the mid-range concentration, d is the estimated response at infinite

concentration, and C_{mAb} is the mAb concentration ($\mu\text{g/ml}$). The mAb concentration (production) of the samples was then determined using the functional inverse of the four-parameter logistic function, as represented in Eq. (7).

$$C_{mAb} = \left(\left(\frac{a-d}{Abs_{450}-d} \right) - 1 \right)^{\frac{1}{b}} \times c \quad (7)$$

Additionally, the mAb productivity of each sample was calculated according to Eq. (8).

$$\text{Productivity}(\text{pg/cell/day}) = \frac{C_{mAb}/C_{\text{cell/ml}} \times 10^6}{t} \quad (8)$$

where t is the time of production in days.

For assay comparison, both the average mAb production and average mAb productivity during the 15 days of culture were determined.

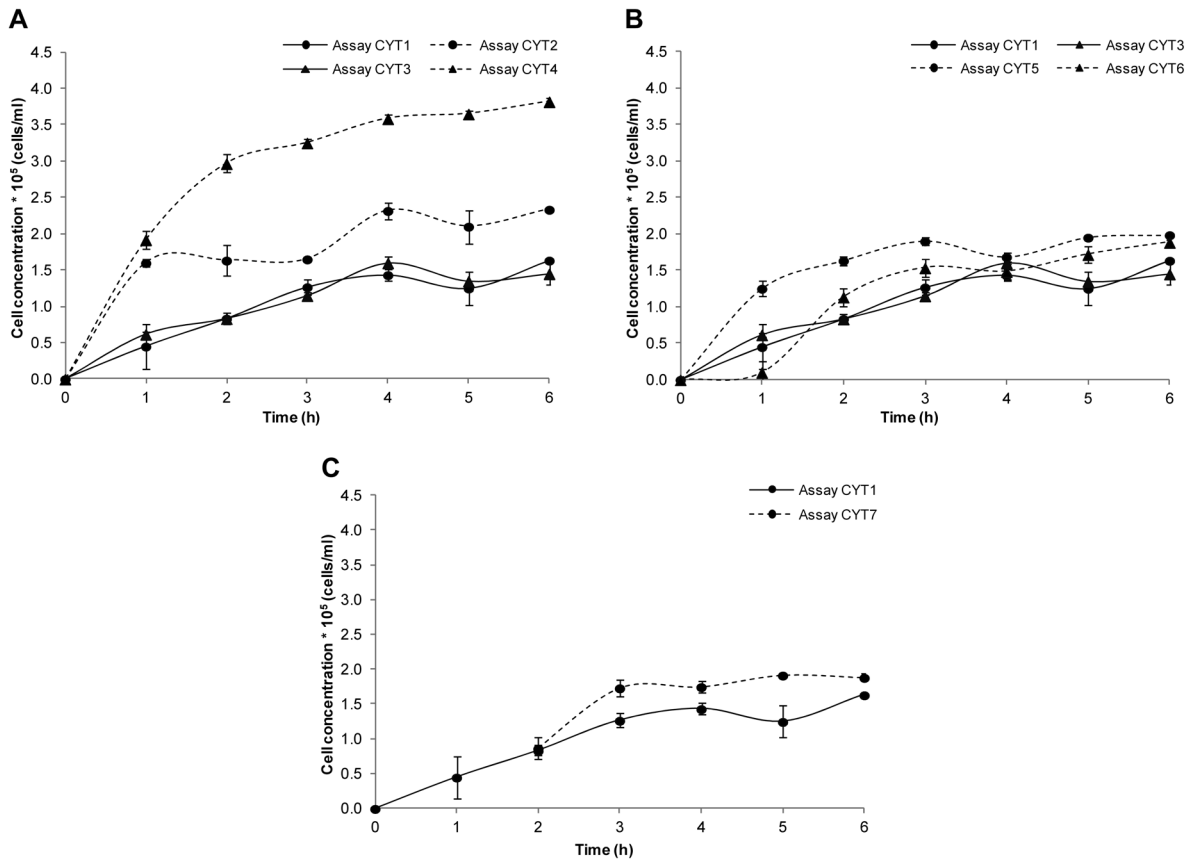


Fig. 1. Evolution of cell adhesion to Cytodex 3 microcarriers during the first 6 h of the 5 ml culture (initial phase of adhesion) in vented tubes.

Comparison of culture conditions: (A) initial cell concentration and rocking mechanism; (B) rocking speed; and (C) initial volume. Assay CYT1: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT2: pulse followed by continuous rocking, 60 rpm, 4×10^5 cells/ml, 3 g/l carriers, total volume; CYT3: continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT4: continuous rocking, 60 rpm, 4×10^5 cells/ml, 3 g/l carriers, total volume; CYT5: pulse followed by continuous rocking, 40 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT6: continuous rocking, 40 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT7: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, half volume.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, ver. 19 (IBM). Results of the production and productivity were assessed using one-way ANOVA with Bonferroni's test, for a confidence level of 95%.

Results

Cultures in Vented Conical Tubes

Cell growth evaluation: phase of initial cell adhesion.

The ability of cells to adhere to the microcarriers was evaluated for each assay, with results shown in Figs. 1 and 2 for Cytodex 3 and CultiSpher-S, respectively. Generally, no relevant differences were observed between the different assays of each microcarrier, with the exception of CUL4

and particularly CYT4. Comparing both microcarriers, it should be noted that the levels of initial CHO-K1 cell adhesion were generally faster and higher in Cytodex 3 cultures.

Cell growth evaluation: phase of cell proliferation.

The ability of cells to proliferate in the microcarriers was evaluated for each assay, and the results are shown in Figs. 3 and 4 for Cytodex 3 and CultiSpher-S, respectively. As observed during the phase of initial cell adhesion, results of the cell proliferation phase demonstrated better growth performances in Cytodex 3 cultures. Comparing the different assays, the culture conditions that seemed more favorable for CHO-K1 cell proliferation were those of CYT2 and CYT3 for Cytodex 3, and CUL6 for CultiSpher-S.

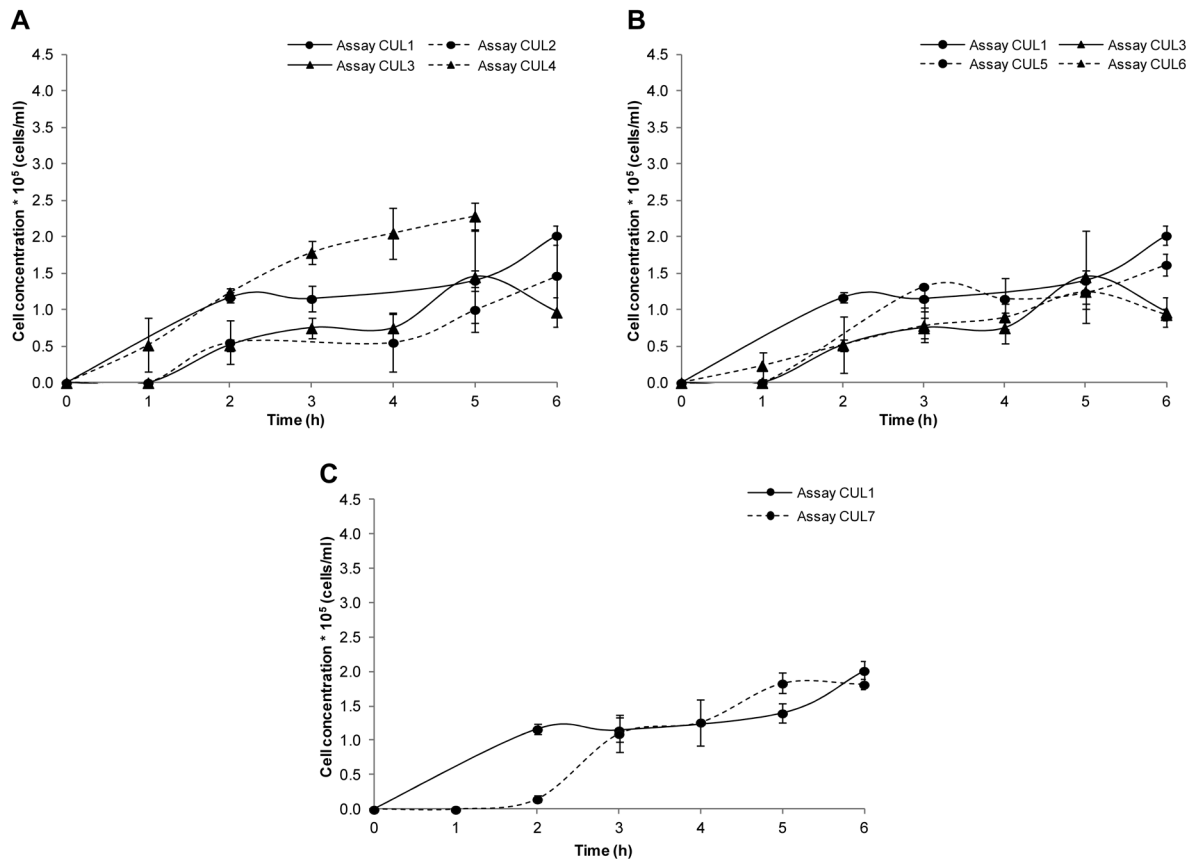


Fig. 2. Evolution of cell adhesion to CultiSpher-S microcarriers during the first 6 h (initial phase of cell adhesion) of the 5 ml culture in vented tubes.

Comparison of culture conditions: (A) initial cell concentration and rocking mechanism; (B) microcarrier concentration; and (C) initial volume. CUL1: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 1 g/l carriers, total volume; CUL2: pulse followed by continuous rocking, 60 rpm, 4×10^5 cells/ml, 1 g/l carriers, total volume; CUL3: continuous rocking, 60 rpm, 2×10^5 cells/ml, 1 g/l carriers, total volume; CUL4: continuous rocking, 60 rpm, 4×10^5 cells/ml, 1 g/l carriers, total volume; CUL5: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 2 g/l carriers, total volume; CUL6: continuous rocking, 60 rpm, 2×10^5 cells/ml, 2 g/l carriers, total volume; CUL7: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 1 g/l, half volume.

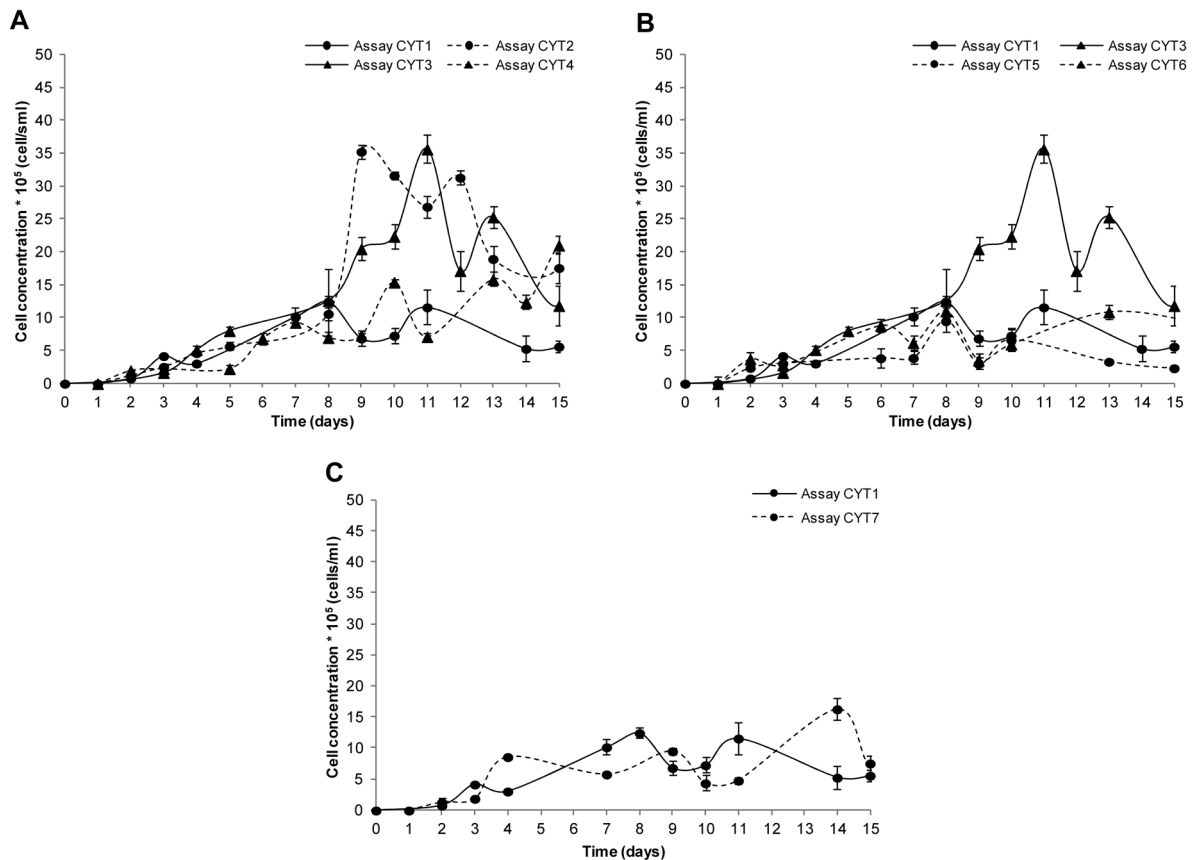


Fig. 3. Evolution of cell proliferation in Cytodex 3 microcarrier cultures of 5 ml in vented tubes.

Comparison of culture conditions: (A) initial cell concentration and rocking mechanism; (B) rocking speed; and (C) initial volume. CYT1: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT2: pulse followed by continuous rocking, 60 rpm, 4×10^5 cells/ml, 3 g/l carriers, total volume; CYT3: continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT4: continuous rocking, 60 rpm, 4×10^5 cells/ml, 3 g/l carriers, total volume; CYT5: pulse followed by continuous rocking, 40 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT6: continuous rocking, 40 rpm, 2×10^5 cells/ml, 3 g/l carriers, total volume; CYT7: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l carriers, half volume.

During the cultures, the microcarriers were visualized microscopically to verify the possible occurrence of structural modifications. Images representative of the changes observed are shown in Fig. 5. Whereas Cytodex 3 kept an apparently unchanged and intact structure during the culture, CultiSpher-S showed a tendency to disintegrate over time, with complete destruction of most carriers by the end of the culture, simultaneously causing the release of cells.

Evaluation of mAb production. The performance of microcarrier cultures at each set of conditions was compared in terms of average mAb production and average mAb productivity, as shown in Table 4. Considering the results of mAb production, Cytodex 3 generally provided superior

levels, whereas CultiSpher-S achieved higher productivities. For the aim of mAb production, the culture conditions providing best results with the CHO-K1 cells were assays CYT1 and CYT2 for Cytodex 3 and assays CUL2 and CUL4 for CultiSpher-S. These assays were therefore scaled-up for further analysis in 20 ml cultures performed in shake flasks.

Cultures in Shake Flasks

Cell growth evaluation. The two sets of conditions that provided higher production levels in the 5 ml cultures performed in vented tubes for both Cytodex 3 and CultiSpher-S microcarriers were scaled-up to 20 ml cultures in shake flasks. Results of cell proliferation are shown in Fig. 6.

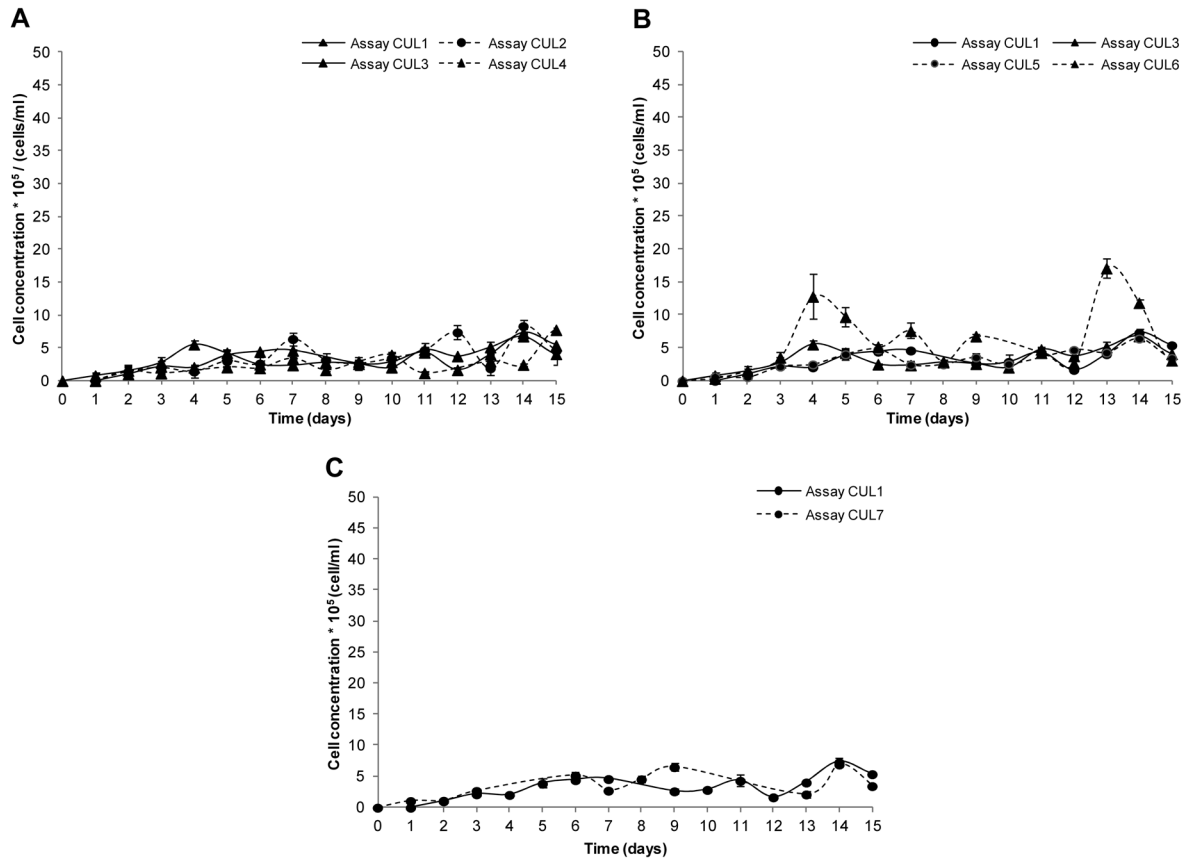


Fig. 4. Evolution of cell proliferation in CultiSpher-S microcarrier cultures of 5 ml in vented tubes. Comparison of culture conditions: (A) initial cell concentration and rocking mechanism; (B) microcarrier concentration; and (C) initial volume. CUL1: pulse followed by continuous rocking, 60 rpm, 2×10⁵ cells/ml, 1 g/l carriers, total volume; CUL2: pulse followed by continuous rocking, 60 rpm, 4×10⁵ cells/ml, 1 g/l carriers, total volume; CUL3: continuous rocking, 60 rpm, 2×10⁵ cells/ml, 1 g/l carriers, total volume; CUL4: continuous rocking, 60 rpm, 4×10⁵ cells/ml, 1 g/l carriers, total volume; CUL5: pulse followed by continuous rocking, 60 rpm, 2×10⁵ cells/ml, 2 g/l carriers, total volume; CUL6: continuous rocking, 60 rpm, 2×10⁵ cells/ml, 2 g/l carriers, total volume; CUL7: pulse followed by continuous rocking, 60 rpm, 2×10⁵ cells/ml, 1 g/l, half volume.

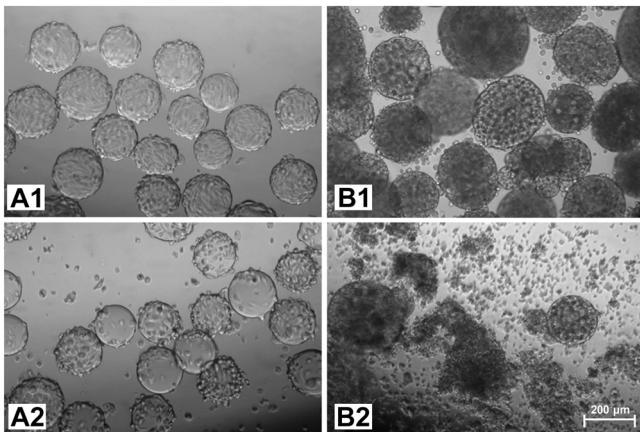


Fig. 5. Microscopic images of (A) Cytodex 3 and (B) CultiSpher-S structures at the (1) beginning (day 2) and (2) end of the culture (day 15).

Results from the shake-flask cultures confirm the better growth performances of CHO-K1 cells in Cytodex 3 microcarriers, already observed for the culture in the vented tubes. Additionally, cells demonstrated similar behaviors for the two sets of conditions assayed for both Cytodex 3 and CultiSpher-S microcarriers.

Evaluation of mAb production. The mAb production and productivity levels achieved in the 20 ml cultures in shake flasks for both Cytodex 3 and CultiSpher-S microcarriers are shown in Table 5. Results show that Cytodex 3 cultures provided the highest mAb production levels, whereas CultiSpher-S cultures achieved the highest productivities. Furthermore, as observed for cell proliferation, the mAb production and productivity levels were similar for the two sets of conditions assayed for both Cytodex 3 and CultiSpher-S cultures.

Table 4. Values of average monoclonal antibody production and average productivity of Cytodex 3 (CYT) and CultiSpher-S (CUL) microcarrier cultures of 5 ml in vented tubes, achieved with the set of conditions of each assay.

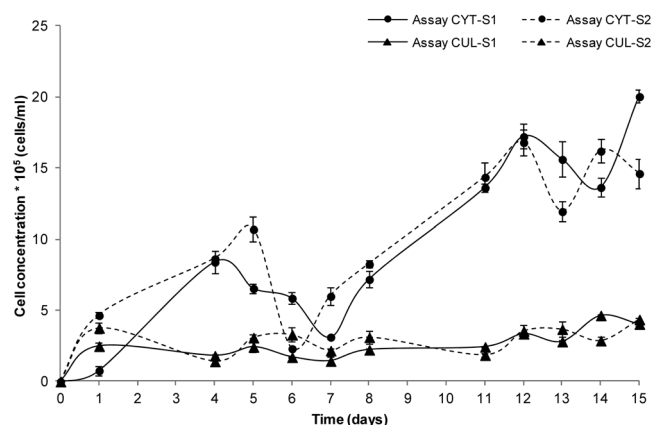
Assay	Average production ($\mu\text{g/ml}$)		Average productivity (pg/cell/day)	
	Cytodex 3	CultiSpher-S	Cytodex 3	CultiSpher-S
CYT1, CUL1	$2.03 \pm 0.67^{\text{ab}}$	1.11 ± 0.35	$2.57 \pm 1.38^{\text{c}}$	$3.48 \pm 0.79^{\text{de}}$
CYT2, CUL2	$1.81 \pm 0.34^{\text{b}}$	1.26 ± 0.49	1.26 ± 0.99	$3.23 \pm 0.71^{\text{de}}$
CYT3, CUL3	$1.49 \pm 0.20^{\text{b}}$	0.94 ± 0.23	0.84 ± 0.35	$2.54 \pm 0.53^{\text{e}}$
CYT4, CUL4	1.61 ± 0.31	$1.85 \pm 0.44^{\text{f}}$	1.51 ± 0.30	$6.67 \pm 2.34^{\text{e,g}}$
CYT5, CUL5	1.39 ± 0.33	1.21 ± 0.31	$3.89 \pm 1.17^{\text{h}}$	$3.00 \pm 0.51^{\text{de}}$
CYT6, CUL6	1.56 ± 0.59	1.10 ± 0.48	2.16 ± 0.94	1.56 ± 0.51
CYT7, CUL7	1.12 ± 0.32	0.94 ± 0.37	1.75 ± 0.66	1.55 ± 1.08

Significant differences ($p < 0.05$): ^ain comparison with CYT7; ^bin comparison with the corresponding assay in CultiSpher-S (CUL1, CUL2, CUL3); ^cin comparison with CYT3; ^din comparison with CUL6 and CUL7; ^ein comparison with the corresponding assay in Cytodex 3 (CYT1, CYT2, CYT3, CYT4, CYT5); ^fin comparison with all the other CultiSpher-S assays except CUL2; ^gin comparison with all the other CultiSpher-S assays; ^hin comparison with CYT2, CYT3, CYT4, and CYT5.

Discussion

In the present work, the use of macro- and microporous carriers for cell growth and mAb production by CHO-K1 cells is discussed. A main concern for the establishment of an efficient process of microcarrier culture is to assure the full colonization of the available surface [8, 38] from the very beginning of the culture [6]. This limits the number of unoccupied beads at the end of the culture, therefore providing higher cell yields. To improve this initial cell

adhesion to the microcarriers, different parameters can be manipulated, such as the rocking mechanism and speed, cell concentration, microcarrier concentration, and initial culture volume that were assessed in this study. For Cytodex 3, the results obtained (Fig. 1) showed that the cell adhesion is slightly improved when using a higher cell concentration (Fig. 1A, CYT1 vs CYT2; CYT3 vs CYT4), particularly when using continuous rocking. The use of continuous rocking proved to be advantageous only for the higher cell concentration (CYT2 vs CYT4; CYT1 vs CYT3), which is interesting since it is reported that the use of pulse rocking during the early attachment stage might improve the rate and proportion of cells attaching to microcarriers [6] by providing more time for cells to settle in their

**Fig. 6.** Evolution of cell proliferation in Cytodex 3 (CYT) and CultiSpher-S (CUL) 20 ml cultures in shake flasks.

CYT-S1: pulse followed by continuous rocking, 60 rpm, 2×10^5 cells/ml, 3 g/l Cytodex 3 carriers, total volume; CYT-S2: pulse followed by continuous rocking, 60 rpm, 4×10^5 cells/ml, 3 g/l Cytodex 3 carriers, total volume; CUL-S1: pulse followed by continuous rocking, 60 rpm, 4×10^5 cells/ml, 1 g/l CultiSpher-S carriers, total volume; CUL-S2: continuous rocking, 60 rpm, 4×10^5 cells/ml, 1 g/l CultiSpher-S carriers, total volume.

Table 5. Values of average monoclonal antibody production and average productivity of Cytodex 3 (CYT) and CultiSpher-S (CUL) microcarrier cultures of 20 ml in shake flasks, achieved with the set of conditions of each assay; and of typical adherent culture in T-flasks and suspended culture in shake flasks.

Assay	Average production ($\mu\text{g/ml}$)	Average productivity (pg/cell/day)
CYTODEX 3		
CYT-S1	2.03 ± 0.51	1.84 ± 0.61
CYT-S2	2.04 ± 0.41	1.57 ± 0.23
CULTISPHER-S		
CUL-S1	0.97 ± 0.37	3.99 ± 0.60
CUL-S2	0.99 ± 0.35	3.54 ± 1.01
Adherent culture (T-flask)	0.54 ± 0.11	0.21 ± 0.04
Suspended culture (shake flask)	1.26 ± 0.42	1.20 ± 0.67

surface. It is possible that the continuous stirring might have enhanced the contact between cells and microcarriers, improving adhesion, and that this effect was more pronounced in the presence of a higher number of cells. On the other hand, pulse rocking might cause settling of cells in layers over the microcarriers, preventing each other from adhering to the carriers.

The literature also suggests that the adhesion to microporous carriers like Cytodex 3 may benefit from lower rocking speeds, for similar reasons as pulse rocking [6]. However, the level of CHO-K1 cell adhesion to Cytodex 3 did not show significant improvement at a lower rocking speed, although providing a slightly faster adhesion (Fig. 1B, CYT1 *vs* CYT5; CYT3 *vs* CYT6).

The initial volume of culture is another important parameter to consider when improving initial cell adhesion to microcarriers. The use of a reduced initial volume, while maintaining the amount of cells and microcarriers of the full-volume assays, may provide a greater chance of contact between cells and microcarriers and improve the conditioning effects of the medium [6]. However, the results obtained demonstrate that the use of half the initial volume (Fig. 1C, CYT7) did not particularly benefit cell adhesion to Cytodex 3 microcarriers, resulting in final levels of cell adhesion (at 6 h of culture), similar to those obtained with a full volume (CYT1).

Regarding cultures with the macroporous CultiSpher-S carriers, the combination of rocking mechanism and initial cell concentration (Fig. 2A) seemed to affect cell adhesion, with pulse and continuous rocking being favorable, combined respectively with low and high inoculum concentrations. Furthermore, two different carrier concentrations were tested (1 and 2 g/l) as advised by the manufacturers. It was observed that the increase in microcarrier concentration was not advantageous, since both concentrations gave similar levels of initial cell adhesion (Fig. 2B, CUL1 *vs* CUL5; CUL3 *vs* CUL6). Additionally, and as observed with Cytodex 3, the reduction of the initial culture volume (Fig. 3C, CUL1 *vs* CUL7) did not improve cell adhesion.

Comparing Cytodex 3 (Fig. 1) and CultiSpher-S (Fig. 2), it is clear that higher levels and faster initial cell adhesion were obtained with Cytodex 3. The characteristics of the surface and the construction material of the microcarriers (Table 1) may be a primary factor leading to the differences observed between the two types assessed. Cytodex 3 has a microporous and more uniform structure, and is composed of a dextran matrix with a collagen coating that benefits a rapid cell adhesion, particularly of CHO cells, and prevents cell detachment. For its turn, the more porous structure of

CultiSpher-S and its uncoated gelatin matrix may not be so favorable for the adhesion of CHO cells. It is also interesting to note that a higher variability between samples (standard deviations) was observed in CultiSpher-S cultures. This may result from the wider range of particle diameter of CultiSpher-S carriers compared with Cytodex 3, as indicated by the manufacturers (Table 1) and confirmed by microscopic observation (Fig. 5).

Following the phase of initial adhesion, cells begin to grow and proliferate in the microcarriers. The goal in this phase of cell proliferation is therefore to improve cell growth and mAb production. Consequently, the ideal culture parameters might be different from those optimized in the initial phase of culture. For example, during cell proliferation, rocking should be continuous in order to provide good nutrient and oxygen transfer to the cells, so the assays initiated with pulse rocking were switched to a continuous methodology after the first 6 h of culture.

Concerning Cytodex 3 cultures, and similar to what was observed in the phase of initial adhesion, the rocking mechanism and the cell inoculum seemed to have the strongest impact on the course of the culture (Fig. 3A). However, it is interesting to note that the conditions resulting in better initial cell adhesion to Cytodex 3 were not the same as those providing the best proliferation profiles (CYT2 and CYT3). This suggests that as long as a good level of cell adhesion to the microcarriers is assured during the first stage of the culture, small differences in these levels will not have a critical impact on the course of the culture. The rocking speed also had a clear influence on cell proliferation, with the highest value (60 rpm) encouraging cell growth (Fig. 3B, CYT1 *vs* CYT5 and CYT3 *vs* CYT6), probably by providing better oxygen and nutrient circulation. On the other hand, and as expected, by not affecting initial cell adhesion to the microcarriers, the reduction of the initial volume of culture also had no impact in the proliferation phase.

Concerning CultiSpher-S cultures, Fig. 4 shows similar proliferation profiles for all conditions tested, with only CUL6 (Fig. 4B) standing out. In this assay, the higher carrier concentration (2 g/l) provided an increased surface for cell growth, and the use of a continuous rocking methodology from the very beginning of the culture might have assured a good nutrient and oxygen circulation through the macroporous structure of CultiSpher-S, enhancing cell proliferation inside these carriers.

Comparing Figs. 3 and 4, it is clear that Cytodex 3 cultures had the highest levels of cell proliferation (particularly CYT2 and CYT3), as already observed for the levels of initial cell adhesion. Indeed, the average growth rate of the

CHO-K1 cells in Cytodex 3 was around 0.04/h, slightly superior to the rate of the cells growing in normal suspended culture (0.03/h), whereas CultiSpher-S cultures had a lower rate of 0.02/h.

Additionally, culture longevity was also much better with Cytodex 3 than with CultiSpher-S carriers, which started to disintegrate after 10-15 days of culture (Fig. 5), whereas Cytodex 3 remains unchanged for at least 30 days (data not shown). The extended culture longevity of Cytodex 3 and the higher levels of proliferation are probably related to the collagen-coating that delays the detachment of the cell sheet, which eventually occurs in long-term culture on uncoated surfaces [37]. Furthermore, the gelatin-based matrix of CultiSpher-S is degradable by enzymes used for culture monitoring (*e.g.*, trypsin), most likely making it more susceptible to degradation by other enzymes released during the culture.

In both microcarrier cultures, but particularly in CultiSpher-S carriers, fluctuations of cell growth were observed. These fluctuations were present in all assays and replicates, and were not correlated with sampling errors, since at least two samples were taken at each sampling point. A possible explanation for this behavior may be pH fluctuations in the medium causing cell detachment from the microcarriers, since a rapid consumption (acidification) of the culture medium was observed even with low cell concentrations and daily medium changes.

In this work, it was also an aim to maximize the mAb yield, which may not be directly related to cell proliferation. Indeed, observing data from Table 4, and comparing with Figs. 3 and 4, it is clear the assays with the greatest levels of production were not necessarily the ones with better cell proliferation. Although higher cell concentrations were achieved for assays CYT2 and CYT3 for Cytodex 3, higher mAb yields were obtained with assays CYT1 and CYT2. However, statistically, the levels of production in Cytodex 3 cultures were similar in almost all assays, with significant differences ($p < 0.05$) observed only between the highest (CYT1) and the lowest (CYT7) producing assays. For CultiSpher-S, although CUL6 had the best proliferation profile, culture conditions defined in assays CUL2 and CUL4 resulted in improved mAb production, with CUL4 being significantly ($p < 0.05$) superior to all assays except CUL2. Results from mAb productivity reinforce the data for mAb production, for CultiSpher-S, with CUL4 standing out ($p < 0.05$). However, the productivity data in Cytodex 3 cultures diverged from production, with CYT5 being the assay with higher productivities ($p < 0.05$), demonstrating that having better productivities does not always translate

into improved productions, so the better culture conditions are those that balance both productivity and cell growth.

It is interesting to note that rocking seems to be the primal parameter affecting production in Cytodex 3 cultures. A pulse followed by continuous rocking of 60 rpm provided the best results for both inoculum concentrations tested (CYT1 and CYT2). On the other hand, production in CultiSpher-S cultures seemed to be more affected by the inoculum concentration, with 4×10^5 cells/ml providing the highest mAb yields (CUL2 and CUL4). The difference observed is most likely related to the microcarrier structure. Whereas the solid structure of Cytodex 3 limits cell growth to its surface and therefore exposes cells to the impact of the rocking mechanism, the porous structure of CultiSpher-S offers increased protection to the cells against the shear stress caused by rocking, by allowing cell growth inside the pores, and thus cell concentration becomes the prevalent factor.

Comparing both microcarriers, the levels of mAb production were generally higher for Cytodex 3 cultures, although significant differences ($p < 0.05$) were only encountered for assays CYT1, CYT2, and CYT3. In contrast, productivities were generally higher for CultiSpher-S, with significant differences ($p < 0.05$) observed for assays CUL2, CUL3, and CUL4. This indicates that the macroporous carriers (CultiSpher-S) potentiated mAb production, but did not benefit cell growth, resulting in final levels of production that were inferior to Cytodex 3. Therefore, these results reinstate the need to manipulate culture conditions so that a balance between cell growth and mAb productivity is found in order to maximize mAb yields.

The culture conditions that provided highest mAb yields were scaled-up for 20 ml cultures in shake flasks. The conditions included pulse followed by continuous rocking at 60 rpm with cell inoculums of 2×10^5 cells/mL (CYT-S1) or 4×10^5 cells/ml (CYT-S2) for Cytodex 3; and an inoculum of 4×10^5 cells/ml and 1 g/l microcarriers with pulse followed by continuous rocking (CUL-S1) or continuous rocking (CUL-S2) for CultiSpher-S (Table 3). The shake-flask cultures (Fig. 6) confirmed that Cytodex 3 microcarriers are more adequate for CHO-K1 cell proliferation, achieving cell concentrations up to five times superior to CultiSpher-S microcarriers, due to the reasons previously discussed (structure and physical properties of the carriers). On the other hand, no notable differences were encountered in cell growth for the two sets of conditions tested for both Cytodex 3 and CultiSpher-S microcarriers.

Observing Fig. 6 in comparison with Figs. 3 and 4, it is noted that the levels of cell proliferation achieved for

Cytodex 3 in shake flasks are similar in both CYT-S1 and CYT-S2, but compared with their vented tube counterparts (CYT1 and CYT2, respectively), there is an increase in the former and a decrease in the latter. For CultiSpher-S, cell proliferation is similar between the shake-flask assays (CUL-S1 and CUL-S2) and also identical to the values obtained in the vented tube assays (CUL2 and CUL4). These observations demonstrate that shake flasks seem to attenuate the differences observed in cell growth between assays performed in the same carriers, which are more notorious in the vented tube experiments.

The comparison of mAb production obtained in shake flasks and in vented tubes (Table 5 *vs* Table 4) shows lower levels of production in shake flasks for CultiSpher-S, particularly for assay CUL-S2, a consequence of a lower productivity of cells growing in these carriers. For Cytodex 3, there was a slight increase of mAb production for CYT-S2 in comparison with CYT2, due to a somewhat higher cell concentration and productivity. Comparing mAb production between both types of microcarriers, Cytodex 3 showed once again to be significantly favorable compared with CultiSpher-S microcarriers. Indeed, although cells growing in the macroporous CultiSpher-S achieved higher productivities ($p < 0.05$), the lowest cell concentrations resulted in lower final mAb yields. For both Cytodex 3 and CultiSpher-S, there were no significant differences between assays concerning either mAb production or productivity.

To conclude, the present study supports microcarrier culture as a viable and favorable alternative to the standard adherent and suspension culture modes, with simple operation, easy scale-up, and significantly higher levels of mAb production. Indeed, the use of Cytodex 3 microcarriers for the culture of CHO-K1 cells gave mAb productions significantly higher ($p < 0.05$) than those obtained with adherent culture performed in 75 cm² culture T-flasks (0.54 ± 0.11 µg/ml) and with suspended culture performed in 250 ml shake flasks (1.26 ± 0.42 µg/ml).

The results obtained demonstrated that although it is critical to assure a good level of initial adhesion to the microcarriers, there is no need for a refined optimization of this stage. On the other hand, it is of utmost importance to find a set of culture conditions that achieve a balance between cell proliferation and mAb productivity for optimized mAb yields. It was found that each microcarrier tested had a different culture parameter that appeared critical for the success of its culture. From all the conditions assayed in this study, rocking mechanism and cell inoculum stood out for their strongest impact on culture performance, with

rocking mechanism being critical for Cytodex 3 and cell inoculum for CultiSpher-S.

Comparing both microcarriers assayed, Cytodex 3, with a microporous structure and collagen-coated dextran matrix, proved to be a better choice than the macroporous gelatin-based CultiSpher-S carriers for the culture of CHO-K1 cells, with regard to cell proliferation, mAb production, and culture longevity. Specifically, Cytodex 3 provided twice the mAb production levels of CultiSpher-S (2.04 µg/ml against 0.99 µg/ml).

Additionally, the culture vessel also seemed to affect microcarrier culture performance. Vented tubes are a good choice for early optimization of the cultures, but do not eliminate the need for optimization of culture conditions at each stage of the scale-up process, since microcarrier cultures will behave differently in vessels of different shapes.

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