Microsupport with two-dimensional geometry (2D-MS)

5. EDTA-mediated detachment of CHO-K1 cells and VERO cells from MicroHexTM

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Abstract

CHO-K1 and VERO cells have been grown on MicroHexTM, a polystyrene-based microsupport with two-dimensional geometry and the consistency of their growth kinetics were established. These cells have been detached by exposure to buffered ethylenediaminetetraacetic acid (EDTA) followed by controlled mechanical shear to yield well-isolated cells suspended in EDTA. Neutralisation of the EDTA followed by restoration of the chemical composition of the growth medium causes detached CHO-K1 cells to display unaltered growth kinetics.

Introduction

With the exception of blood cells, the 200 or so different cell types which constitute our organism, do not grow as isolated entities. For the majority of them, there is a need to be anchored to a surface.

As of today, mass production at industrial scale of anchorage-dependent cells (ADCs) is a discontinuous batch process involving repeated cycles of trypsinisation followed by neutralisation of the enzyme. Much care must be exercised especially during trypsinisation in order to avoid clumping of the microsupport and the formation of aggregates of different sizes containing varying number of entrapped cells.

This work is aimed at avoiding the use of trypsin, relying only on ethylenediaminetetraacetic acid (EDTA) and controlled mechanical shear to detach anchorage-dependent CHO-K1 cells from MicroHexTM, a polystyrene-based tissue culture grade microsupport with two-dimensional geometry. VERO cells were also tested to evaluate their sensitivity to EDTA in order to try and generalise the

detachment protocol to other cell lines of biotechnological interest.

Materials and methods

Materials

The CHO-K1 and VERO cells used in these experiments were obtained from the ECACC and Cambrex (formerly Biowhittaker, ref no 85021005 and CRL-1934, respectively). They were propagated in Nunc triple stage flasks (total surface area of 500 cm²) filled with 150 ml DMEM-F12 (v/v) (Gibco, ref no 21331-046) growth medium supplemented with 2 mM glutamine and 10% newborn calf serum (Gibco ref no 16010-159). Tissue culture grade MicroHexTM (760 cm²/g) was obtained from Nunc A/S (Nalge Nunc International, Roskilde, Denmark). The experimental conditions leading to the determination of the optimised detachment of the cells from MicroHexTM were performed in spinner flasks (Techne, Cambridge, UK).

Diagram 1. Double dual pinch valves.

To determine the growth kinetics of CHO-K1 at a specific surface area of $10 \text{ cm}^2/\text{ml}$ culture in 1-l cultures, we used Applikon (Schiedam, The Netherlands) 3-l (total volume) glass jacketed bioreactors equipped with two small scooping impellers one above the other and the double dual pinch valves (DDPV), an home-made sedimentation-based perfusion device. The reactor was equipped with a pH and pO_2 control.

The core of DDPV (Diagram 1) was made out of two stainless steel sedimentation tubes ST1 and ST2, each composed of a 15-mm diameter inner tube through which the cell-laden MicroHexTM suspension was alternatively aspirated (peristaltic pump PP2) and an outer concentric tube (25 mm wide), used to introduce fresh growth medium in the culture via peristaltic pump PP1. PP1 worked intermittently during 1 min during each 5-min cycle while PP2 worked continuously.

There were two liquid dual pinch valves (LDPV) whence the coining DDPVs, LDPV1 and LDPV2 which set the injection of fresh medium in the culture and the retrieval of cell-laden MicroHexTM suspension out of the bioreactor, respectively.

Each pinchvalve worked antagonistically, that is, when LDPV1 pinched (closed) line 1, line 2 was open and vice versa. The same held true for LDPV2, lines 2' and 1'.

Perfusion was composed of successive 5-min periods during which, the cell-laden MicroHexTM suspension was alternatively pumped via the inner tube of ST1 or ST2, the principle being that while the suspension was pumped via one of the sedimentation tubes, the microsupport had time enough to sediment in the other one. Considering the sedimentation tube at rest, that is, that one in which the microsupport was sedimenting, fresh growth

medium was forcibly pumped into the culture via the latter outer concentric tube during 1 min starting 3 min after the beginning of each 5-min cycle.

Methods

Spinner cultures

Experiments performed in spinner flasks (maximal volume: 350 ml) were conducted at a specific surface area of 10 cm^2 MicroHexTM per millilitre of culture volume in 150-ml growth medium using an inoculum of 25,000 cells/ cm^2 of microsupport. The stirring speed was chosen so as to just keep the microsupport in suspension (20–25 rpm). At day 1, after a complete change of medium, the stirring speed was increased to 30–35 rpm. Growth kinetics were followed by daily sampling the culture and counting the nuclei following crystal violet/citric acid digestion of the cells–microsupport complex. Two milliliters of homogeneous cells-laden microsupport suspension were delivered into a 15-ml pre-weighted (P_0) capped plastic centrifuge tube and weighted (P_1) tube. The spent growth medium above the gravity-sedimented microsupports was discarded by suction and 5 ml of phosphate buffered saline (PBS) carefully added so as to just resuspend the microsupport pellet avoiding any shear that might detach cells from the microsupports. The just sedimented microsupports, rid of PBS were resuspended in a final volume of 2 ml crystal violet solution and the tube weighted once again (P_2). Isolated nuclei (N) were counted in a Bürker counting chamber either after 2 h incubation at 37°C either after 2, 3 days at room temperature, care being taken to avoid naked microsupport sliding under the coverslip of the counting chamber. The cell concentration ρ (cells/ml culture) was calculated from the relation: $\rho = 10,000 \times N \times (P_2 - P_0)/(P_1 - P_0)$.

Three to four days after inoculation, upon reaching confluence, gravity-sedimented MicroHexTM laden with cells were washed once with PBS and suspended in PBS–EDTA (all operations took place at 37°C) for varying periods and in varying concentrations of the chelating agent (0.5–2 mM) in final volumes one-half to one-eighth of the initial culture volume. The cells were detached by four successive passages of the cells/PBS–EDTA suspension through the 2 mm orifice of a 25-ml plastic pipette to yield naked MicroHexTM and isolated cells suspended in PBS–EDTA. EDTA was then

neutralized with one-100th the PBS–EDTA suspension volume of 50 mM MgSO_4 and the normal chemical composition of the complete growth medium restored by the addition in succession of newborn calf serum to 5% v/v, glutamine solution to 2 mM and five times concentrated DMEM-F12 (v/v) growth medium. Cells number and viability were determined by trypan blue staining. The percent recovery was calculated from the number of cells released by EDTA, expressed in function of the total number of attached cells as determined by enumerating the nuclei following incubation of a sample of cell-laden MicroHexTM with Crystal Violet.

Serial subcultivations

The method used for testing the quality of the used (“conditioned”) MicroHexTM, that is, its ability to support successive rounds of multiplication was as follows: five independent cultures were simultaneously initiated in spinner flasks. Every other day, to cope with the potential exhaustion of nutrients, a 100% medium exchange with fresh medium took place in all five spinners after gravity sedimentation.

Spinner 1 (control culture) was sampled daily and had its growth curve determined. Having reached confluence, spinner 1 was interrupted and discarded.

The cells from the confluent cultures 2–5 were then detached in one-half their culture volume of PBS–EDTA (0.5 mM). The naked MicroHexTM from cultures 2–5, thus all conditioned once (1C-MicroHexTM), were extensively washed with PBS to eliminate all traces of EDTA and remove any cells remaining potentially trapped between the microsupport particles.

1C-MicroHexTM derived from each of the four cultures 2–5 were again mixed with cells (25,000 cells/cm² – 10 cm²/ml) and growth allowed to proceed until confluence was again reached in all four cultures (by visual inspection).

At that moment, PBS–EDTA detachment yielded MicroHexTM support having undergone two rounds of “conditioning”: 2C-MicroHexTM. Repeating this succession of manipulations yielded 3C-, 4C- and 5C-MicroHexTM.

Reactor cultures

For 1 l culture volumes (3 l total capacity bioreactor) and a specific surface area of 10 cm²/ml, the following inoculation protocol was used: $2.5 \cdot 10^8$ CHO-K1

cells and 13.16 g MicroHexTM (total microsupport surface area of 10,000 cm²) were suspended in 1 l growth medium and attachment allowed to proceed during 24 h at 45 rpm. At that time, agitation was stopped and unattached cells in suspension were removed following careful suction of the spent growth medium (free of microsupport) above the gravity-sedimented cell-laden MicroHexTM. The volume of the culture was then brought back to 1 l with fresh growth medium and agitation resumed at 50 rpm. Forty eight hours after inoculation, agitation was increased to 55 rpm, perfusion was started at 1 culture volume per day and kept constant throughout the experiment.

The protocol used to detach the cells from the microsupport started with the incubation for 15 min at 37°C of PBS-washed cell-laden MicroHexTM in 500 ml (half the culture volume) of PBS–EDTA (0.5 mM).

Compressed air at 0.3 bar was then applied on the surface of the suspension to force the latter to exit from the bioreactor and pass through a stainless steel filtration supporting plate (diam. 16.5 cm) perforated with multiple 2 mm holes, contained in an hermetic housing (Balvinox, Mont-St-André, Belgium).

This was considered passage 1. The whole mixture was collected in a 1 l collecting bottle. Pressure in the bioreactor was then released and applied now on the collecting bottle, forcing the suspension to pass a second time through the holes of the stainless steel filtration supporting plate. This series of operations was repeated once, making the microsupport to pass four times through the holes to finally accumulate in the bioreactor together with detached cells.

Results

The first series of experiments using CHO-K1 cells as a reference, all conducted in spinners aimed at determining the optimal conditions for detachment.

Progressively increasing the time of exposure to PBS–EDTA (0.5 mM), led to a progressive increase in cell recovery, levelling after some 15 min of exposure (Table 1). The length of incubation at 37°C was not extended beyond that period since gain in recovery would have been marginal and also for fear of jeopardising the cell viability.

The results of the experiments aimed at evaluating the influence that PBS–EDTA with EDTA concentrations of 0.125, 0.250 and 0.500 mM over 15 min, had on cell recovery and viability are summarised in Table 2. Whatever the concentration of phosphate buffered EDTA was used, the cell viability remained stable at 93–95%. However for EDTA concentrations below 0.25 mM, recovery dropped from 73–77% down to 47%.

The next series of experiments were devised to see whether, keeping the total amount of EDTA constant (volume of PBS–EDTA times concentration of EDTA was constant), detachment could be realised by decreasing the volume of PBS–EDTA solution and proportionally increasing the concentration of EDTA. The results of such experiments are given in Table 3. They show that keeping the total amount of EDTA to which the cell-laden MicroHex™ were exposed constant, that is,

Table 1. Influence of the duration of the exposure to PBS–EDTA (0.5 mM) on cell recovery and viability

Time (min)	Number of cells collected/ml ($\times 10^{-6}$)	Recovery (%)	Cell viability (%)
0	0.695	27	74
3	1.47	57	92
5	1.43	56	87
8	1.72	67	92
15	1.85	72	95

Table 2. Influence of the concentration of EDTA

EDTA concentration (mM)	Number of cells collected/ml ($\times 10^{-6}$)	Recovery (%)	Cell viability (%)
0.500	2.2	73	95
0.250	2.3	77	93
0.125	1.4	47	94

varying the PBS–EDTA solution volume between one-half to one-eighth of the original culture volume and simultaneously increasing proportionally the concentration of the chelator from 0.5 to 2 mM, made no difference with respect to cell recovery or cell viability.

Notwithstanding the constant and high cell viability obtained after exposure of cell-laden MicroHex™ to EDTA concentrations ranging from 0.5 to 2 mM, experiments whose results were summarised in Table 3 did not tell us anything about the capacity of cells detached at each of these EDTA concentrations to resume normal growth after restoration of the normal chemical composition of the complete growth medium (see Materials and methods).

Figure 1 shows three control growth curves obtained from separate experiments using as inoculum CHO-K1 cells detached from tissue culture flasks by the PBS–EDTA (0.5 mM) method. As observed from the almost overlapping curves, consistency was good. Figure 1 also shows that cells from the third control culture, detached in half, one quarter or one-eighth the culture volume of PBS–EDTA at EDTA concentrations of 0.5, 1 and 2 mM EDTA, respectively, displayed growth kinetics identical with those of the three control cultures upon restoration of the normal chemical composition of the complete growth medium.

For serial cultivation, knowing the number of times that the same lot of MicroHex™ could be used without affecting the growth kinetics of the cells, is a prerequisite which should be established beforehand. Using the experimental protocol described in the section ‘Methods’, Figure 2 shows that 1C- to 5C-MicroHex™ whatever the number of conditioning cycles they underwent, upon inoculation with PBS–EDTA detached cells, displayed the same growth kinetics as the control culture performed with commercially available unused tissue culture grade MicroHex™.

Table 3. Influence of the volume of the PBS–EDTA solution during EDTA-mediated cell detachment at constant total amounts of EDTA

Volume of PBS–EDTA (ml)	Ratio: vol PBS–EDTA/culture vol	EDTA concentration (mM)	Number of cells collected/ml ($\times 10^{-6}$)	Recovery (%)	Cell viability (%)
50	1/2	0.5	2.42	87	94
25	1/4	1	2.28	82	97
12.5	1/8	2	2.27	82	95

Figure 1. Growth kinetics of CHO-K1 cells detached from MicroHex™-carriers after exposure to various concentrations of EDTA (150 ml culture volume; 10 cm²/ml MicroHex™). Three independent 150 ml cultures numbered 1–3 (controls) were performed and their growth kinetics were determined as described in the section ‘Methods’ (spinner cultures). Three days after inoculation, 90 ml of the cells-laden MicroHex™-carriers suspension from culture 3 were divided in three 30 ml portions and cells separately detached in one-half (15 ml), one-fourth (7.5 ml) or one-eighth (3.75 ml) the volume of the portion of 0.5, 1 and 2 mM buffered EDTA, respectively. The cells from each portion were used to inoculate three independent 150 ml cultures numbered 31–33 whose growth kinetics were then determined. ●, ■, ▲ (plain lines): control cultures 1, 2, 3 respectively; □, △, ○ (dotted lines): cultures 31 (1/2 vol EDTA 0.5 mM), 32 (1/4 vol EDTA 1 mM), 33 (1/8 vol EDTA 2 mM), respectively.

Based on the results obtained from the 150 ml spinner flasks, the next series of experiments aimed at scaling up to 1 l using a 3 l bioreactor. In order to maintain constant the chemical composition of the growth medium throughout growth, discontinuous medium changes were replaced by continuous perfusion with fresh medium (1 l/day) using the DDPV system. Three independent experiments performed under these conditions established the consistency of the process (Figure 3).

Three days after inoculation, the cell concentration reached in 150 ml spinner flask (Figure 2) was slightly higher than the corresponding value obtained in 1 l perfused bioreactor (Figure 3).

Figure 5A (control) shows the degree of confluency reached between days 3 and 4 by the cultures whose growth kinetics are displayed in Figure 3: cells were so tightly packed together, that individual cell-laden MicroHex™ began to firmly stick together. Following detachment with PBS–EDTA and mechanical shear, cells were released from the microsupport (Figure 5B): they existed as individual entities in suspension, with no apparent aggregates.

Figure 2. Growth kinetics of CHO-K1 cells on used (“conditioned”) MicroHex™-carriers (150 ml culture volume; 10 cm²/ml MicroHex™). CHO-K1 cells grown on unused commercially available MicroHex™-carriers had their growth kinetics compared with those obtained using MicroHex™-carriers onto which cells grew and were subsequently detached with buffered EDTA (0.5 mM) up to five times (1C- to 5C-MicroHex™-carriers) as described in the section ‘Methods’ (serial subcultivations). *, □, ▲, ●, △: 1C-, 2C-, 3C-, 4C-, 5C- MicroHex™-carriers, respectively.

Figure 3. Growth kinetics of CHO-K1 cells in a 3-l bioreactor (10 cm²/ml MicroHex™, culture volume: 1 l, perfusion rate: 1 culture volume/day). Three independent cultures numbered 1–3 were performed in a perfused 3-l bioreactor and their growth kinetics were determined. ◇, ■, ▲: cultures 1, 2, 3, respectively.

Very few cells if any resisted the treatment and remained attached onto their microsupport.

Figure 4 shows the growth kinetics of 4 independent cultures of VERO cells conducted in spinner flasks. Here again, as for CHO-K1 cells, the results were reproducible. Cell densities of $1.1\text{--}1.4 \times 10^4$ cells/cm² were obtained consistently. VERO cells exposed for 15 min at 37°C to PBS–EDTA (0.5 mM) detached poorly following several

passages through the 2 mm bore of a 25 ml pipette (data not shown). They could be detached however when the bore diameter was reduced to 1.3 mm (Figure 5C and D).

Figure 4. Growth kinetics of VERO cells on MicroHex™-carriers (150 ml culture volume, 10 cm²/ml MicroHex™). Four independent 150 ml cultures numbered 1–4 were performed and their growth kinetics were determined. △, ■, ▲, □: cultures 1, 2, 3 and 4, respectively.

Discussion

Compared to the propagation at industrial scale of isolated cells in suspension, mass cultivation of ADCs in continuously stirred tank reactors presents such a series of bottlenecks that the general tendency is to try and adapt them to grow in suspension, preferably in serum-free chemically defined growth medium. The adaptation takes a long time, is expensive to perform and more than often when it succeeds – it can also fail – results in cells growing as smaller or larger aggregates. Attempts to mass cultivate such cells at high concentration in (sono) perfused bioreactors is counteracted by the progressively increasing aggregation of already existing aggregates.

The observation by Van Wezel (1967) that ADCs attached and grew on the surface of positively charged dextran-based microbeads kept in suspension in continuously stirred tank reactors not only considerably facilitated the production of this type

Figure 5. Pictures of cell-laden MicroHex™ were taken at confluency (A: CHO-K1/1L culture – corresponding to day 3 of culture 2 (■) in Figure 3; C: VERO/150 ml culture – corresponding to day 4 of culture 4 (□) in Figure 4) and after detachment with EDTA (0.5 mM) and controlled mechanical shear (B: CHO-K1; D: VERO).

of cells but also paved the way to multiple applications in biological sciences. MicroHexTM is a new microsupport characterised by a two-dimensional geometry (hexagonal flakes). It presents a higher surface area-to-volume ratio than microcarriers with three dimensional geometry (microbeads), which allows in theory larger numbers of ADCs to be accommodated per millilitre culture (Miller et al. 1989; Chowdhury et al. 1989; Bio Directory, 2002).

Optimisation of the detachment by EDTA of CHO-K1 cells from MicroHexTM in spinner flasks, delineated the temperature (37°C), time of exposure (15 min), chelator concentration (0.5 mM), mechanical treatment (four passages through the 2 mm bore of a 25-ml pipette) and volume of buffered 0.5 mM EDTA (one-half the volume of the original cell-laden MicroHexTM culture) required to obtain well isolated, high quality cells (95% viability).

In the glass spinner flask, it is the soft gyratory motion of the pendulum with its rounded end which just kept the microsupport in suspension. In the 3 l bioreactor this role was fulfilled by the two scooping impellers one on top of the other. The fact that CHO-K1 cells grew systematically faster in spinner than in bioreactor (compare Figures 1 and 2 with Figure 3) was probably due to the reason that the scooping impellers produced more shear forces than the pendulum.

At all times considered, CHO-K1 cells grew faster in spinner flask than did Vero cells under the same experimental conditions. Duration of the experiments limited to 4 days did not allow us to draw any conclusions concerning their capacity to form more than one layer or to stop growing.

Cells obtained by EDTA treatment of a confluent cell-laden MicroHexTM culture at 10 cm² microsupport/ml culture performed in spinner flasks, yielded 150 ml of isolated cells in suspension at about 2.2×10^6 cells/ml (Table 3), that is, approximately 200 cells per microsupport (100 cells per apposed surface).

Provided the rheology of the culture remained the same (at 10 cm² MicroHexTM/ml culture, it took 24 h to homogeneously inoculate the microsupport, cell-laden microsupport sedimented at a minimum speed of 1 cm/min and proper sedimentation took place in the sedimentation tubes of the DDPV used for perfusion), linear extrapolation of the data obtained at 10 cm² microsupport/ml culture to

50 cm² microsupport/ml culture should give cell concentrations of around 10^7 cells/ml at confluence.

Adhesion is thought to be mediated by ionic interactions (most of the time involving Ca²⁺) and proteins bridges. Depending upon which of these two types of interactions is the strongest, a chelator or a protease (or a mixture of both) will be best suited for detachment. In our hands, the facile detachment of CHO-K1 cells with EDTA suggested that for this cell line, ionic interactions prevailed over protein bridges. Other cell lines behave in the same way (Jones 1991; Merten et al. 1997). It was the inverse situation which prevailed for VERO cells, which were very difficult to detach with EDTA only, and for MDCK which were absolutely resistant to EDTA exposure.

As a result of the action of EDTA on some cell surface components of the cell membrane – cytoskeleton structure, cells become leaky, round and present fewer attachment points a situation which should make them more amenable to detachment (Merten 1999).

At the 1-l scale, EDTA-treated CHO-K1 cells did not detach from MicroHexTM even when the reactor's stirring speed was increased to 350 rpm (data not shown). The same result was observed with CHO-K1 cells attached on cryoresponsive MicroHexTM. The explanation for this phenomenon has been discussed earlier (Kenda-Ropson et al. 2002a,b). Apparently the vortex induced by the scooping impellers in the 1-l culture was unable to generate the desired shearing forces required for detachment. The cells detached however after four passages through the 2-mm large holes of a stainless steel filtration supporting plate under 0.3 bar of pressurised air (controlled mechanical shear), a treatment which mimics the shear-induced mechanical detachment on a laboratory scale using a 2-mm wide bore 25 ml pipette.

MicroHexTM onto which CHO-K1 cells grew and detached afterwards with EDTA were reused as many as five times producing at each growth cycle MicroHexTM tentatively "conditioned" once, twice, up to five times (1C- to 5C- MicroHexTM). CHO-K1 cells detached from T-flasks by the *conventional trypsin-EDTA* detachment procedure or by our method grew equally well on these 5 sorts of MicroHexTM (data not shown). Whether reused "conditioned" MicroHexTM were truly conditioned and covered by an extracellular matrix awaits analysis

by mass spectrometry of the attached proteins and glycosylaminoglycans.

Consistent growth kinetics could be obtained at laboratory scale in spinner flasks at MicroHex™ specific areas of 10 cm² MicroHex™/ml culture (2×10^6 cells/ml culture with about 200 cells/microsupport at confluence) with CHO-K1 and VERO cells. The cells from both cell lines could be detached with EDTA (provided that the mechanical detachment was adapted to the cell line under consideration) without recourse to trypsin, led to obtaining isolated cells in suspension showing a high cell viability. CHO-K1 cells, obtained in this way and regrown on unused or “conditioned” MicroHex™, displayed the same growth kinetics as control cultures obtained with cells detached by the conventional trypsin EDTA procedure. It is not known whether VERO cells behave in the same way and can be subcultured after detachment with buffered EDTA.

It is anticipated that MicroHex™ would be a useful microcarrier for the serial cultivation of CHO-K1 cells and other ADC cell lines grown under static conditions provided that they can be detached with EDTA from polystyrene T-flasks or cell factories.

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