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Microsupport with two-dimensional geometry (2D-MS) 4. Temperature-induced detachment of anchorage-dependent CHO-K1 cells from cryoresponsive MicroHex® (CryoHex)

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Abstract

Tissue culture flasks were activated by electron beam irradiation and subsequently treated with N-isopropylacrylamide to make them cryoresponsive. Leaving such 'cryoflasks' unattended for 10 minutes at room temperature sufficed to almost completely detach the anchorage-dependent cells. MicroHex®, a polystyrene-based tissue culture microsupport with two-dimensional geometry, was handled in the same way to obtain CryoHex, i.e. a cryoresponsive MicroHex from which anchorage-dependent cells could be detached by exposure to low temperature (4–20 °C). Experimental conditions were determined allowing one to detach the cells from small and large microsupport culture volumes. Cells detached from CryoHex by exposure to low temperature displayed a high cell viability and, upon subcultivation on MicroHex®, did not show any alteration of their growth kinetics.

Introduction

Growth of anchorage-dependent cells on an industrial scale is best performed in continuously stirred tank reactors using microsupports (Brown et al. 1985; Van Wezel 1985; Gatot et al. 1998; Zecchini and Smith 1998).

Handling large amounts of microcarriers under good manufacturing practice (GMP) conditions is by no means an easy task. Also, repeated treatment with trypsin in order to detach cells at confluence for amplifying the cultures to production scale, results in decreased cell viability, not to say anything about the potential hazards associated with the potential contamination by adventitious agents such as prions (Dormont 1999).

The advent of polystyrene-based tissue culture microsupports with two-dimensional geometry (MicroHex®, NUNC A/S, Denmark) characterized by a high surface-volume ratio, has led to surface modifications difficult to envisage with polysaccharidic microsupports.

This paper presents the results of experiments performed with such a microsupport grafted with poly(N-isopropylacrylamide), a well-known cryoresponsive polymer, the aim being to detach cells by exposure to low temperature (4–20 °C).

Materials and methods

CHO-K1 cells were obtained from ECACC (Ref. 85021005). They were grown in DMEM-F12 (Gibco 21331-046) containing 10% Newborn Calf Serum (Gibco 16010-159).

NUNC's tissue culture grade 80 cm^2 culture flasks (Ref. 1-53732A) were first irradiated with a 25 Mrad (250 kGrays) electron beam (Power: 10 MeV, Intensity: 100 μA and Dose rate: 10–15 Kgrays/min) using a linear type electron accelerator working with a static mode (from Gent University). These irradiated 80 cm^2 flasks were subsequently grafted (3 h, 60 °C) with 30 ml of a 10 wt.% recrystallized N-isopropylacrylamide (Acros, Ref. 412785000) dissolved in water to yield cryoresponsive tissue culture-grade culture flasks: the CryoFlasks. These CryoFlasks

were inoculated with CHO-K1 cells (1.9×10^6 cells) in 30 ml complete growth medium and incubated at 37 °C in a humidified cabinet gassed with 5% CO_2 in air. Near or at confluence, 15 ml of the spent growth medium was withdrawn and discarded. Leaving the culture flask 10 min at room temperature on the bench made the cells detach spontaneously.

Tissue culture grade MicroHex® was obtained from NUNC (Nalge Nunc International, Roskilde, Denmark – Ref. 139108, 750 cm^2/g). Its characteristics have already been fully described (Gatot et al. 1998). It was derivatised with N-isopropylacrylamide in the same way as tissue culture grade flasks to yield first an electron-beam activated MicroHex and, subsequently, upon incubation with N-isopropylacrylamide, cryoresponsive MicroHex also called CryoHex.

To 75 ml culture in a 250-ml Techne glass spinner vessel, were added 1.1 g (825 cm^2) of sterile (30 min in 70% v/v isopropanol in water) CryoHex and 3×10^7 cells (24.000 cells/cm^2). The resulting suspension was agitated in a humidified cabinet gassed with 5% CO_2 with a stirring speed low enough to just keep the microsupport in suspension (18–20 rpm). After 24 hours, the microsupport suspension was decanted, the unattached cells removed by suction and growth allowed to resume following the addition of 150 ml fresh growth medium. From that moment on, the stirring speed was increased to 30 rpm. Around day 4 after inoculation, twenty-five ml of cells-laden microsupport suspension were sampled from the culture and put in a 50-ml plastic centrifuge tube. Following one wash with 25 ml ice-cold PBS and 20 or 30 minutes exposure to 25 ml of ice-cold PBS with intermittent shaking, the cells were detached by 5–10 aspirations through the 0.2 cm-large bore of a 25-ml pipette. Later it was shown that pipetting cells-laden CryoHex, left 20–30 min in their original growth medium cooled down to 4 °C, produced the same results, making the ice-cold PBS wash redundant.

For 1L culture, the same experimental protocol was followed using ten times more microsupport (11 g) and cells (3×10^8 cells) in complete growth medium using a 3L total capacity continuously stirred tank reactor (Applikon glass jacketed bioreactor equipped with a scooping impeller). During the first 24 hours, the culture volume was kept at 500 ml and the agitation at 30 rpm. Afterwards, the final volume was brought to 1 L by the direct addition of 500 ml of fresh growth medium, the stirring speed being increased from 30 to 80 rpm (no decantation step was used). Upon reaching confluence, the water at 37 °C in the bioreactor's glass jacket was rapidly replaced by water circulating at 4 °C. Thirty minutes later, the stirring speed was increased from 80 to 150 rpm to force the cells to detach. Because cells detached poorly even at this high strirring speed, alternative methods were sought. It was found that very good cell recovery – as inferred by visual inspection – could be obtained by passing vigorously 5 times, in rapid succession, the CryoHex suspension under an air pressure of 0.3 bar through the holes (0.2 cm in diameter) of a stainless steel filtration supporting plate (diameter: 140 mm, from Tami Filtration).

Results

Cells from a confluent culture grown in a CryoFlask (Figure 1A), left unattended at room temperature on the bench for 10 minutes or so, peeled off by themselves as large flakes (Figure 1B). Vigorous though controlled aspiration of this suspension through the 0.2 cm large bore of a 25 ml plastic pipette dissociated the flakes into more or less well isolated cells (Figure 1C). Cells from a confluent culture grown in a non cryoresponsive tissue culture flask did not detach when submitted to the same treatment (Figure 1D).

As compared to anchorage-dependent cells cultivated under static conditions as described above, the next series of experiments aimed at determining whether CHO-K1 cells could attach and grow in agitated suspension on CryoHex and subsequently become detached upon lowering the temperature of the culture.

Figure 2 shows the growth of anchorage-dependent CHO-K1 cells on MicroHex®, only irradiated by an electron beam. Twenty four hours after inoculation (Figure 2A), some cells were still unattached but the majority of such irradiated MicroHex showed cells evenly spread on them. Confluency was reached by day 4 (Figure 2B). These cells-laden irradiated MicroHex, washed with PBS and kept in PBS at 4 °C for 20 (Figure 2C) or 30 (Figure 2D) minutes resisted mechanical detachment.

The same experiment performed with cryoresponsive MicroHex, i.e. CryoHex, showed comparable colonisation (Figure 3A) and growth (Figure 3B). Reasonable detachment of cells from such microsupport occured after only 20 min at 4 °C (Figure 3C) and was complete after 30 min (Figure 3D). Control experiments performed at temperatures higher (37 °C) or lower (20 °C) than the lower critical solution

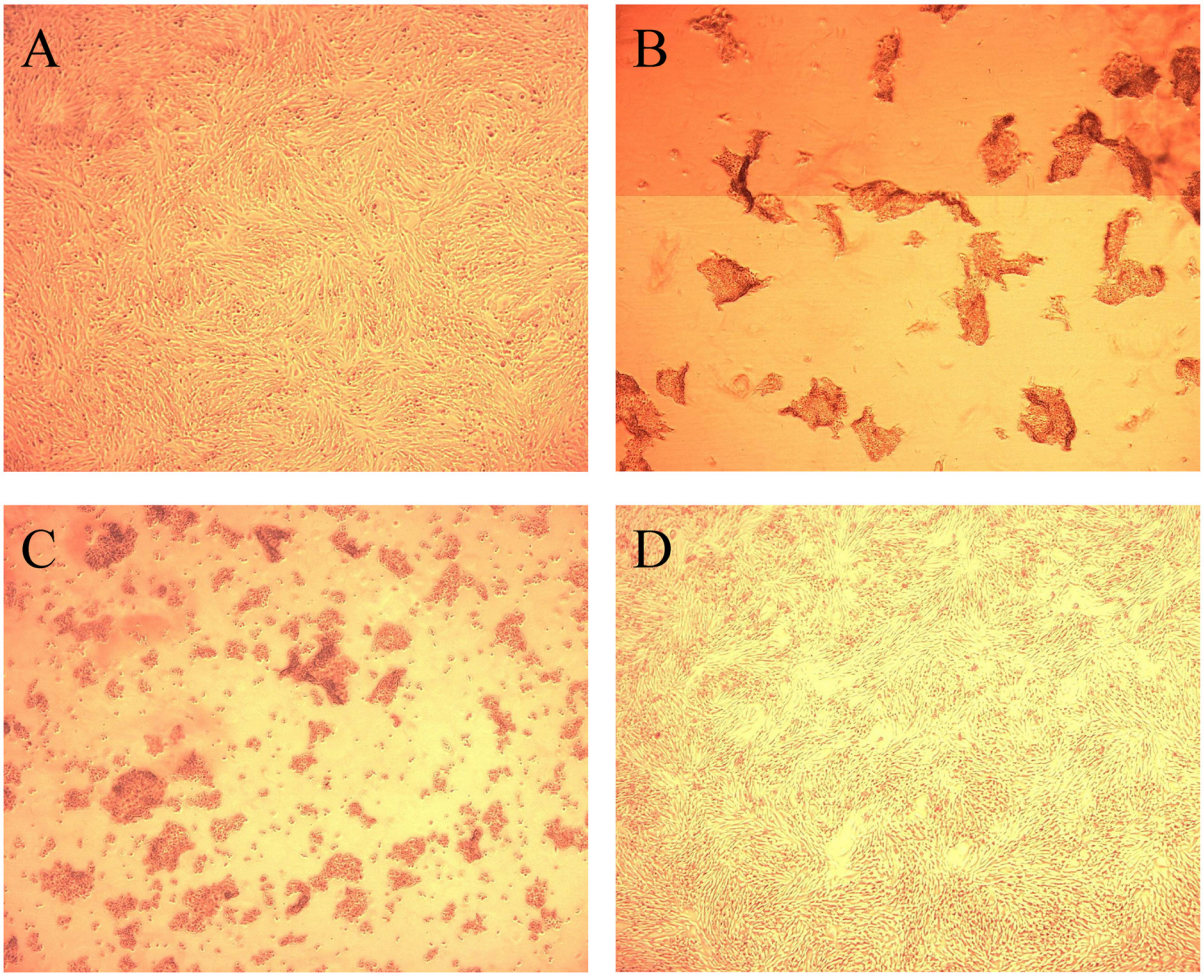

Figure 1. Exposure to low temperatures of a monolayer of anchorage-dependent CHO-K1 grown in a cryoresponsive tissue culture flask. A) CHO-K1 anchorage-dependent cells grown in a cryoresponsive 80 cm^2 tissue culture flask until confluence. B) monolayer of CHO-K1 cells grown in cryoresponsive tissue culture flask left 10 min at room temperature in 15 ml complete growth medium. C) the cells obtained in Figure 1B after mechanical disruption by forced aspiration through a 0.2 cm-wide bore. D) monolayer of CHO-K1 cells grown in tissue culture grade flask (non cryoresponsive) left 10 min at room temperature in 15 ml complete growth medium.

temperature (LCST; i.e., 25 °C for poly (N-isopropylacrylamide) in PBS) showed no detachment (Figure 3E) or cell detachment (Figure 3F), respectively. This method produced individual cells together with flakes whose size varied with the way the cells-laden CryoHex suspension was aspirated through the orifice of the pipette.

Because Techne spinner flasks used in these experiments did not lend themselves to industrial scale up, experiments similar in essence to those already described were conducted at the 1 L scale in a continuously stirred tank reactor. In order to simplify the process, PBS was replaced by complete growth medium during the 30 min exposure to 4 °C. Contrary to our expectations, cells did not detach after 30 min stirring at 0–4 °C. Nor did they, when the duration of the exposure to 4 °C and/or speed of agitation were increased (up to 50 min at 150 rpm – Results not shown). However, provided the culture has not reached confluence, the cells could become quantitatively detached (Figure 4B) by vigorously passing the cells-laden CryoHex suspension (exposed 30 min at 4 °C) through a perforated stainless steel plate, as described in Materials and methods. Cell viability as measured by trypan blue staining, before and after detachment remained good in this case, 98% and 92% respectively. The same treatment applied to cells-laden CryoHex overcrowded with cells (Figure 4C)

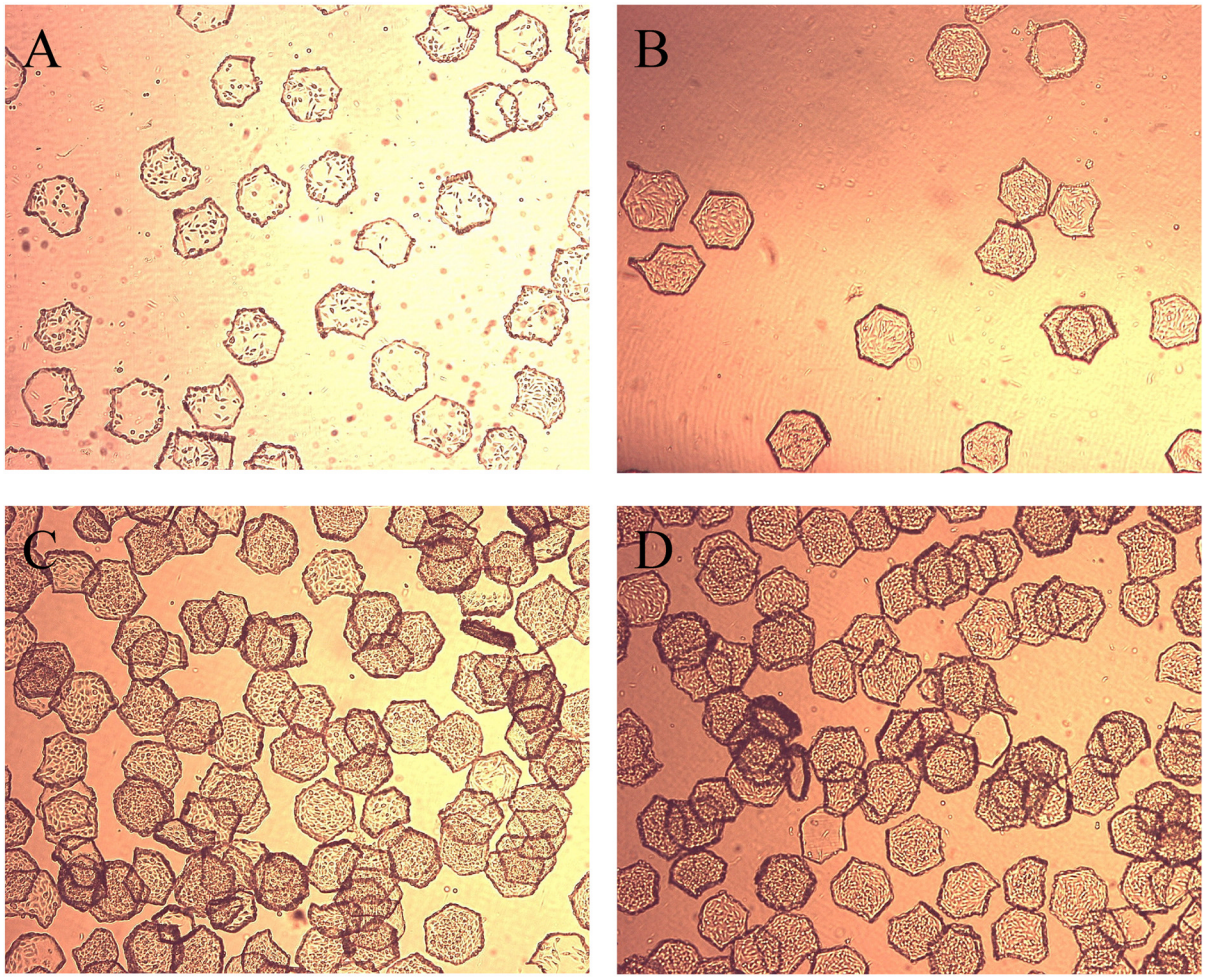

Figure 2. Exposure to low temperatures of cells-laden electron-beam irradiated MicroHex (non cryoresponsive) (Techne spinner vessel). Electron-beam irradiated (non-cryoresponsive) MicroHex was inoculated with anchorage-dependent CHO- K1 cells and the extent of growth recorded 1 day (Figure 2A) or 4 days after inoculation (Figure 2B). Samples from the 4 day-old culture were exposed to ice-cold PBS for 20 min (Figure 2C) or 30 min (Figure 2D) before mechanical detachment by vigorous aspiration.

led to incomplete detachment and the formation of aggregates of large size (Figure 4D). The quality of CHO cells having undergone thermal shock was investigated by subcultivating them on MicroHex®. As shown in Figure 5, their growth kinetics (Figure 5, curve B) did not differ from those displayed by cells detached with trypsin-EDTA (Figure 5, curve A). Colonisation of CryoHex with CHO-K1 cells was however faster on MicroHex® than on CryoHex (Figure 5, curve C).

Discussion

CryoHex is a temperature-sensitive, hexagonally-shaped flat microflake prepared from tissue culture grade polystyrene-based MicroHex® microsupport which completes the list of existing cryoresponsive substrates used to grow anchorage-dependent cells under static conditions such as tissue culture polystyrene dishes (Kwon et al. 2000; Okano et al. 1995; Yamato et al. 1999; Kikuchi et al. 1998; Chen et al. 1998; Kushida et al. 2000) or porous membranes (Kwon et al. 2000; Chen et al. 1998).

To confer cryosensitivity, the experimental procedure arrived at consisted of two phases. In the first one, active sites were generated on the tissue culture grade polystyrene surface by static electron beam irradiation (250 kgrays and a 10 MeV electron source) in an N_2 atmosphere carefully purged from any trace

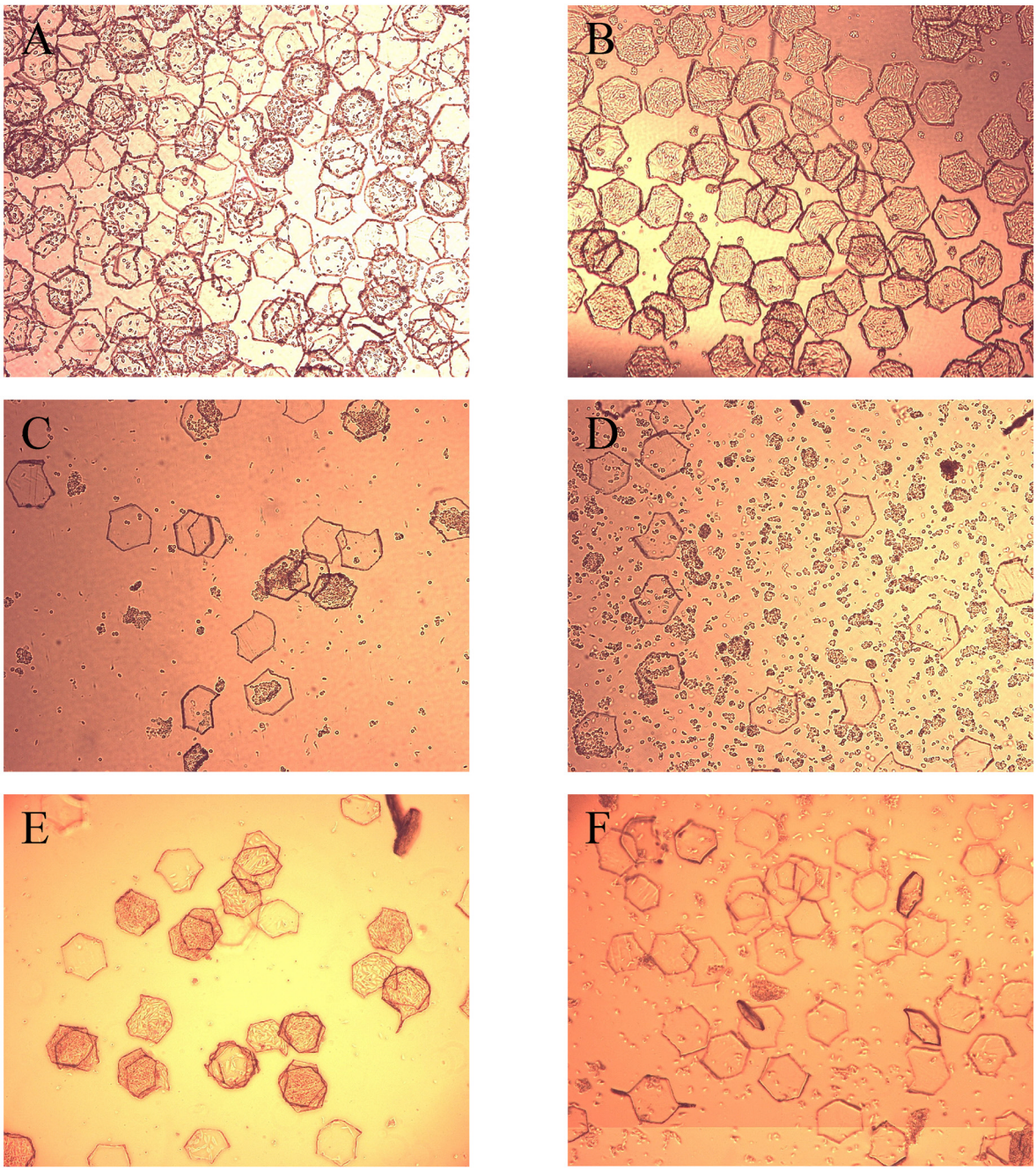

Figure 3. Exposure to low temperatures of cells-laden CryoHex (cryoresponsive MicroHex) (Techne spinner vessel). Same experiment as in Figure 2 using CryoHex (cryoresponsive MicroHex). A) 1 day-old culture. B) 3 day-old culture. C) 3 day-old culture after cryodetachment (20 min in ice-cold PBS). D) 3 day-old culture after cryodetachment (30 min in ice-cold PBS). E) 3 day-old culture. Detachment performed in complete growth medium at 37 °C. F) 3 day-old culture. Detachment performed in complete growth medium at 20 °C.

of oxygen. In the second phase, the activated surface thus obtained was immediately grafted with N-isopropylacrylamide monomer which chain polymerized during a 3 h incubation at 60 °C also in an oxygen-purged N_2 atmosphere, yielding the so called CryoFlask or CryoHex.

Submitting commercially available tissue culture grade flasks to this two-phase activation-graft/poly-

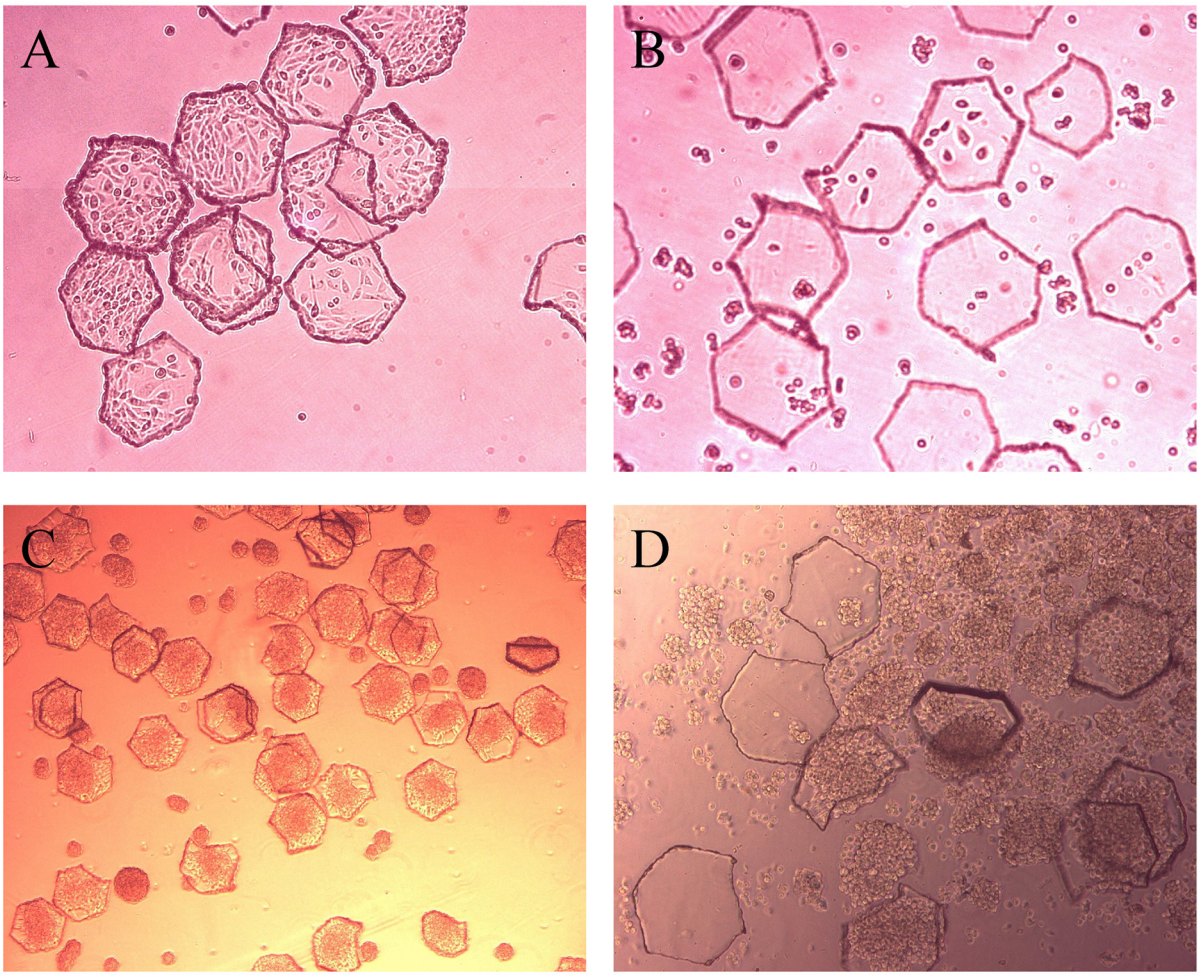

Figure 4. Detachment of cells from cells-laden CryoHex (cryoresponsive MicroHex) at the 1 L culture volume in agitated culture. CryoHex was inoculated with anchorage-dependent CHO-K1 cells and the extent of the growth recorded 4 days (Figure 4A) or 8 days (Figure 4C) after inoculation, i.e. well before or beyond confluence, respectively. On days 4 and 8 (Figure 4B and 4D), 500 ml culture were taken, the volume brought to 1L with ice-cold growth medium, the resulting suspension exposed to 4 °C and then treated as described under Materials and methods. A) 4 day-old culture. B) 4 day-old culture after cryodetachment. C) 8 day-old culture. D) 8 day-old culture after cryodetachment.

merisation derivatisation procedure renders them cryosensitive. Much care should be exercised during the manipulation of CryoFlasks: simply leaving them unattended for 10 min at room temperature sufficed to make the cell monolayer spontaneously peel off as big flakes whose size could slightly be reduced by mechanical disruption (Figure 1).

The next series of experiments aimed at determining the detachment protocol which should be used to detach anchorage-dependent cells grown on CryoHex in agitated cultures.

The results of experiments performed on laboratory scale in agitated cultures using Techne glass spinner flasks showed that CHO-K1 cells attached and grew on electron-beam activated MicroHex as well as they did on MicroHex. As expected, they were not cryoresponsive (Figure 2).

Figure 3 shows that grafting N-isopropylacrylamide monomer on electron-beam activated MicroHex, followed by the polymerization of the latter molecule into poly(N-isopropylacrylamide) cryosensitive chains (phase 2), made it possible for the cells to attach and grow on CryoHex in agitated cultures. Cryodetachment now became observable every time after 20 or 30 min exposure to ice-cold PBS. The observation that cells did not detach from CryoHex at 37 °C but did so at 20 °C definitely established the cryoresponsive character of CryoHex,

excluding the possibility of detachment by mechanical tear.

In an attempt to investigate large scale production, similar experiments were performed at the 1L culture volume using first ice-cold PBS replaced later by spent growth medium when it was observed that cell detachment ocurred equally well in both liquids. Increasing the stirring speed of the bioreactor – an obvious strategy for the biotechnologist – from 80 up to 150 rpm following 50 min exposure at 4 °C did not detach the cells from the CryoHex. It seemed as if the vortex induced by the scooping impeller in the 1L culture was unable to generate the desired shearing forces required for detachment. This observation is reminiscent of the resistance to detachment opposed by CHO-K1 cells anchored onto MicroHex® during Laser Diffracted Particle Sizing at pump/strirrer speeds as high as 1250 rpm (Kenda-Ropson et al. 2001). Cells could nevertheless become detached provided that the pre-cooled (30 min at 4 °C) cells-laden CryoHex were forced to pass 5 times in rapid succession through the 0,2 cm-wide holes of a stainless steel filtration supporting plate, mimicking in fact the mechanical action of the 25 ml pipette. The short time (3 min) required to perform such a detachment system (after the exposure to 4 °C) and having the possibility to increase the area (diameter) of the perforated stainless steel plate makes industrial scale-up straightforward. Cell viability was not affected by this treatment. However the extent of detachment was dependent on the degree of confluence reached by the culture: cells detached poorly from overcrowded CryoHex to form cell aggregates of varying sizes (Figure 4).

The temperature at which anchorage-dependent cells detached themselves from a cryoresponsive surface depended on the nature of the fluid bathing the cells-laden support surface, the type of cell and their metabolism (Okano et al. 1995). In our hands, whenever suspended at 20 °C in complete growth medium or in ice-cold PBS, 20–30 min were required to completely detach CHO-K1 cells. From the biochemist's point of view, keeping the system at 0–4 °C instead of at 20 °C for 30 min, decreased the risks of enzymatic degradation brought about by the highly labile (glyco)protein secreted in the culture medium. For the biotechnologist however, rapidly decreasing the temperature from 37 °C down to 0–4

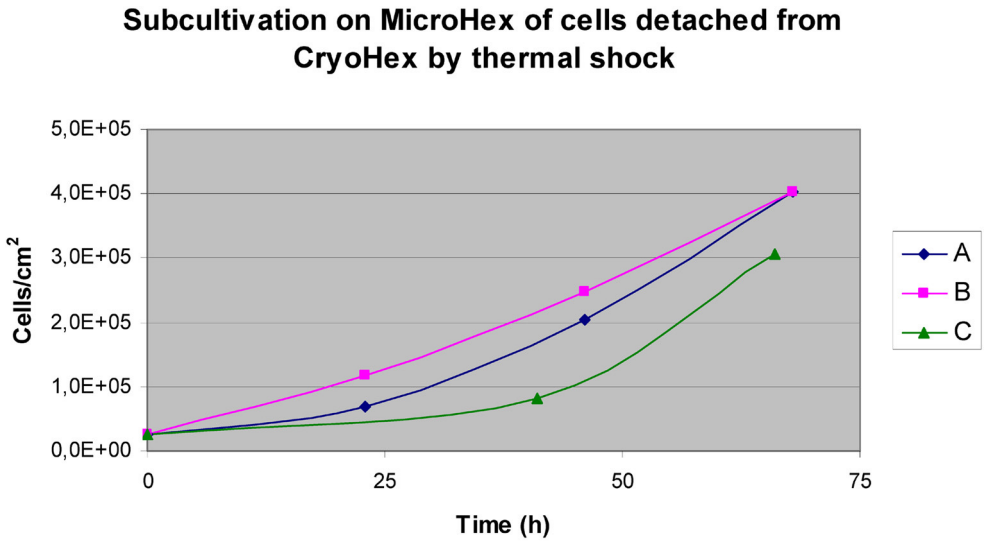

Figure 5. Subcultivation on MicroHex® of cells detached from CryoHex by exposure to low temperature. CHO cells grown on CryoHex in a Techne spinner vessel were detached in the cold with a pipette as described in Materials and methods and used to inoculate MicroHex®. Concomitantly another culture was started also on MicroHex® this time using cells detached from a tissue culture grade flasks with trypsin-EDTA. Both cultures had their growth kinetics then determined. A) growth kinetics of CHO cells detached from tissue culture grade flasks by trypsin-EDTA and subcultivated on MicroHex® (Control). B) growth kinetics of CHO cells detached from CryoHex by exposure to 4 °C and subcultivated on MicroHex®. C) growth kinetics of CHO cells detached from tissue culture grade flasks by trypsin-EDTA and subcultivated on CryoHex.

°C must set technological problems especially with large-capacity bioreactors (10.000 L) in a GMP environment. Obviously, lowering the temperature from 37 °C down to 20 °C would cause less trouble at the expense maybe of the quality of the secreted biological more liable to undergo degradation. Under such experimental conditions, degradation could nevertheless be kept to a minimum if the anchorage-dependent cells are grown in perfused continuously stirred tank bioreactors (Gatot et al. 1998).

Figure 5 shows – as expected – that cells detached from CryoHex by exposure to low temperature could be subcultivated and grew on MicroHex® as well as cells obtained by the current trypsin-EDTA protocol, underpinning the absence of physiological trauma.

In conclusion, CryoHex constitutes a useful means to easily propagate and recover anchorage-dependant cells on a laboratory scale and probably on an industrial scale in stirred tank reactors. CryoFlasks and CryoHex also makes it possible to propagate anchorage-dependent cells for the preparation of membrane receptors for which protease-based detachment methods cannot be used.

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