Test of a novel transparent floating carrier by recombinant CHO cells expressing sIL6 receptor protein

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Abstract

We describe a carrier with some unusual properties. “Cellrafts” increase yields from adherent cells in conventional static T150 flasks, by floating multilayer strips of transparent film at the oxygen-rich surface of unstirred medium. This new technique allowed microscopic inspection of cell growth inside the carriers during bulk culture. Individual carriers could be picked out for subculture of selected colonies. A novel surface treatment by hypochlorite/uv allowed recycling of used carriers. Cellrafts’ open-deck structure facilitated trypsinization with 90% release as viable single cells from bulk carriers. Macro size (10 mm by 1 mm) enables retention in flask by a coarse sieve insert in its neck, facilitating separation of product cells or media. Residual cells in carriers regenerated repeated harvests without need for reseedling. Carriers were tested with shear-sensitive CHO clones expressing soluble human IL6 receptor (sIL6R). Control was monolayer bulk culture on trays. Floating multilayer cultures remained viable longer than monolayers, had higher cellular activity of protein expression, and were less serum dependent (resembling cells on porous carriers). Purity and anti-sIL6R binding were identical to control product. Cellrafts were also tested in a small spinner vessel, but for litre batches this proved less convenient than in T-flasks. Though yields are low compared to well established porous carrier technology (spinner or packed bed) static transparent carriers might provide transitional scaleup from normal cytogenetics laboratory culture.

Introduction

The “cellraft” carrier design attempts to bridge a gap between static culture in T-flasks as used to grow up clones for protein expression, and stirred carriers for bulk production. Microscopy of cells in situ is routinely required during clone development, and trypsinization is essential for scaleup. Commercially available porous macro carriers of 2–7 mm diameter (Looby and Griffiths 1988; Kadouri et al. 1992) are not transparent, and trypsinized cells remain trapped in tortuous pores. Most carriers also require a stirrer or pump to supply oxygenated medium. The unstirred “oxygen diffusion boundary layer” in static tissue culture is only 1–3 mm (Maroudas 1974). Folkman and Greenspan (1975) emphasize the critical impor-

tance of a short path (< 2 mm) to oxygenate a cell matrix by diffusion alone.

To improve oxygenation and trypsinization we developed (with Anthony Saunders of Minerva Technology, Woking GU21 4QR UK) a porous carrier called Porocell (Handa-Corrigan et al. 1998) which floated at the oxygen-rich surface of unstirred medium. Porocell also had smooth, wide pores that released viable trypsinised cells - but was not completely transparent. A previous design, short ribbons of film (Kadouri et al. 1988) was highly transparent but could only be used in packed bed with pump.

Here we combine both concepts (floating carrier and transparent ribbon) to make multilayer “cellrafts”. We describe the development of cellrafts, and report tests with fragile, sparsely confluent CHO

clones genetically modified to express soluble Interleukin 6 Receptor protein (sIL6R). In our cytogenetics laboratory, trial quantities of product from shear sensitive clones were normally obtained by monolayer bulk culture in static Nunc trays. We compare control monolayer growth and sIL6R protein expression with multilayer culture on cellrafts in flat static flasks; also on cellrafts in a stirred vessel.

Materials and methods

sIL6 receptor assay

Microwell plates (Immunosorb, Nunc) were coated with anti-IL6R monoclonal antibody to which IL6-Receptor was bound and assayed as described (Novick et al. 1991).

sIL6 receptor purification

Medium, concentrated 10 fold by a 10 kD molecular filtration unit (Amicon) was passed through an Affigel column (Biorad) coupled with IL6-receptor specific monoclonal antibody. Eluted fractions were checked for band purity by SDS electrophoresis on 10% polyacrylamide gel with Coomassie blue stain, and for protein specificity by ELISA.

Cell culture under standard conditions

Cell clones CA5 and IID3, from CHO cells transfected with sIL6 receptor expression vector (Oh et al. 1996) were maintained in medium F12/DMEM with 10 mM HEPES, 100 $\mu\text{g ml}^{-1}$ Streptomycin, 100 units Penicillin, 150 $\mu\text{g ml}^{-1}$ Proline and 5% Fetal Calf Serum. Serum free medium was F12/DMEM with insulin and transferrin 5 mg l^{-1} , selenium 0.1 mg l^{-1} plus Yeast Extract Ultrafiltrate 50X (Sigma Y4375) and lipids (1000X, Sigma L5146) added in that order. Standard bulk monolayer culture was on 600 cm^2 Nunc trays. Seed cultures were split 1:4 at confluence (0.8 E5 cells cm^2). Trypsinised cells were counted by haemocytometer with Trypan blue to verify that more than 95% of the cells were viable. For production on trays, cells from 9 cm dishes were seeded at 20 million per tray in 50 ml medium with 10% FCS. At confluence (50 million cells per tray) the medium was changed to 75 ml with 2% FCS. Medium was harvested and replaced with a fresh 2% FCS medium

every 2–3 days until the culture deteriorated (about four batches per tray).

Fabrication of cellrafts

The basic format is a stack of transparent film 15–20 micron thick, assembled as 10 layers 30–100 micron apart. Spacing was measured by microscopy edge-on (as in Figure 1b).

The film is a flame-perforated polypropylene from Protos Packaging (Sharpness GL13 UK, polypropfilms@aol.com). It has two advantages. Flame-oxidation aids cell adhesion. The perforations (0.4 mm diam) form large pores in the film, and their raised rims (dark rings Figure 1a) act as spacers between the layers. Cellraft carriers were handmade at first, by spotwelding the layers together with a needle-tipped soldering iron, then cutting with scissors. Later they were webmade by Osmotek (Rehovot, www.osmotek.com). Individual carriers measured 6–12 mm long by 1–2 mm wide by 0.5–1 mm thick. The planes were shaped long and narrow (like glider wings) to provide a lift force that allowed these macrosized carriers to be stirred at the same low speed as for microcarriers (30–60 rpm). Narrow width also speeded oxygen diffusion, and made a short flowpath for trypsinised cells to wash across the cellraft's open decks. 2 g (2,000 cm^2) occupied 15–20 ml bulk volume. Theoretical cell density for these sparse cells (8E4 per cm^2) would be about 8E7 cells g^{-1} carrier dry wt. Since actual yield was less than half, 2 g was rated as 1 Roller Bottle unit (800 cm^2). When the T150 flask was laid flat, carriers spread out to float in a zone less than 2 mm thick at the air water interface. Result: a layer of "tissue" (water plus immobilized cells) thin enough to minimize the risk of oxygen starvation (Folkman and Greenspan 1975).

Surface treatment

The first carriers were surface treated dry, with handheld Tesla coil as described previously (Maroudas 1993; Maroudas et al. 1995) till contact angle was ≤ 30 degrees. Angle was measured by shaking samples in a flask with distilled water, then examining edge-on by microscope for tiny airbubbles which flatten (analogous to cells spreading on a treated surface) from nearly spherical (high contact angle) to nearly flat. Finally we evolved a new method: wet photo chemical oxidation. Carriers in T-flask or stain-

Figure 1. A. Bulk cellrafts *in situ* X3. Large rings are the raised rims of 400 micron pores. Small white dots are cells. Angular edges are corners of multilayer foils. B. Fluorescence with Acridine Orange. Edge-on view X10. Dark strips are edges of the 15 micron foil multilayers. Bright areas are cell monolayers and multilayers. C. AO fluorescence normal view X30, showing bright (yellow) mitotic figures. D. Giemsa stain, oil immersion X100. Dark areas show nuclei and spread cytoplasm.

less steel dish were immersed in phosphate buffered sodium hypochlorite (1% at pH8) and irradiated under a uv lamp (40 or 80 watt tube) at a distance of 1–5 cm for 4–12 hours until correct contact angle is attained and PB-hypochlorite is completely reduced to form harmless PB-Saline (confirmed by starch/iodide test).

Reuse of carriers

Cellrafts were recycled after soaking in 1% sodium hypochlorite (or household bleach diluted 1:3) overnight at 60 °C (or a week at room temp) to dissolve cellular matter, and washing 3–6 times with water then PBS plus Methyl Red pH7, in flasks with fitted sieve. Sterilization was by leaving wet carriers in flasks under uv hood overnight; or by autoclaving in PBS.

Culture on cellraft carriers

Seeding

Seed cells were applied in the minimum of medium. 10–25 million cells in 10 ml of inoculum were added to 20 ml cellrafts, prewashed with medium, in a T150 flask with vented cap. Cells were distributed evenly amongst the multilayers by tilting the flask. The flask was then laid flat in incubator and inspected every half hour (turning flask upside down occasionally to seed both top and bottom surfaces) until cell adhesion was complete (3–5 hours). A slow rocker (1 rpm) was helpful but not indispensable.

Growth

Fifty ml of medium was added for initial growth, and thereafter 100–200 ml per batch feed. Flasks were incubated in 5% CO₂, and the medium changed when acid. Cells were examined under microscope for

confluence. If acidity became excessive the flask was removed from CO₂ incubator to warmroom. If the medium then became too alkaline, the flask was returned to incubator or its vent was partially masked with foil.

Harvesting soluble products

Batches of media were harvested with the help of a screen insert that held back the cellrafts while product was being emptied from the vessel. We cut a 2 mm mesh nylon strainer 2.5 cm diam, with bendable wings to make a push-fit in the neck of T150 flask. Ready-cut strainers were later punched by Osmotek. Even without a strainer, most of the cellrafts could be retained by decanting gently, because these long, straight shapes tended to “logjam” as they approached the narrowing neck of the flask.

Harvesting cells

Cellrafts were washed and trypsinized as for dish culture, with trypsin/EDTA pro rata to the increased number of cells (30 million per gram cellraft). The cell suspension was separated from carriers by gently shaking the flask, then pouring out through sieve in neck.

Spinner culture

4 g of autoclaved cellrafts in 50 ml of 5% FCS medium was poured into a 500 ml spinner vessel through one of its two 1” sideports. A sieve was push fit in the neck of the other port. Seed was 20 million in minimal liquid, and the vessel was rotated every half hour to distribute cells evenly (inspected by laying vessel on microscope). For growth, 300 ml of medium was added and stirred at 30–60 rpm, sufficient to mobilize the floating carriers without dragging them down into stirrer blades. Medium was changed and IL6 receptor assayed every 3 days.

Microscopy

For inverted microscopy *in situ* the medium had to be less than 5 mm deep, otherwise the carriers floated too high to focus. Samples were withdrawn with forceps or a perforated spatula, then washed and stained with bulb pipette in 12 mm wells. Acridine orange stain was used as a growth indicator (Ringerts et al. 1970; Maroudasand and Wray 1985).

Results

Microscopy

With routine low power inverted microscope, x4–x20 unstained cells were clearly visible in the bulk culture *in situ*, by focussing through the successive layers of any individual cellraft (Figure 1a).

Fluorescent stain proved especially suitable for focussing through successive multilayers. Cellrafts allow an unusual edge-on view, which revealed individual cells in sagittal projection, as well as monolayer and thicklayer cell growth (Figure 1b). Bright yellow AO fluorescence indicated active nuclei, with chromosomes and mitotic figures clearly visible (Figure 1c).

With absorption stains, confluent multilayers became opaque but separate foils were transparent, revealing nuclear and cytoplasmic detail under oil immersion at 100 x (Figure 1d). By scratching the surface it could be shown that cells had spread on both sides of the film layers.

Production

Cell production

A 150 cm² T-flask plus 2 gram of cellrafts was seeded with a single inoculum of 1 E7 cells CHO-IID3, in 100 ml medium with 5% FCS. Near confluence the flask was placed on a rocker and trypsinised weekly for a month, to yield four successive cell harvests. (Each new harvest being generated from residual cells of the previous trypsinization).

Trypsin released about 90% of the cells from confluent multilayers: yield 6E7 cells per flask (20% more than from the control tray). About 6E6 cells remained within the cellrafts. The effective growth surface was thus equivalent to an 800 cm² Roller Bottle: $6.6E6 \text{ cells} / 0.8E5 \text{ cells per cm}^2 = 825 \text{ cm}^2$ (of which 150 from T-flask hence 675 cm² from the cellrafts). Bulk cell density was calculated as $6.6 / 20 = 3.3E6 \text{ cells ml}^{-1}$ bulk volume (or $6.6 / 2 = 3.3E7 \text{ cells g}^{-1}$ carrier).

Production of sIL6 receptor in roux bottle

First trial was in a 1 litre glass Roux bottle with cottonwool plug, autoclaved with 50 ml of cellrafts in PBS, and seeded with 15 million cells in 10% FCS. Serum was reduced to 2% in production medium. The Roux culture was left unstirred in the incubator for two months, batch fed twice weekly by gentle de-

Table 1. Production of cells in T150 flask

Cell yield from 600 cm ² control	5 E7
Cell yield from flask + cellrafts	6 E7
Cell total after four repeat harvests	24 E7

cantation of product medium and replacement with 100–200 ml of fresh. Control tray cultures could not be maintained for longer than 2 weeks. Although sIL6-Receptor titre was higher in the trays, the Roux culture lasted longer and eventually yielded 5 times as much product (Table 2).

Production of sIL6R in T-flasks

A set of 20 trays was compared with a set of 6 T-flasks in static incubator culture, using CHO transfect line IID3. The tray cultures remained viable long enough to yield 3 batches per tray on average, while the T-flasks averaged 9 batches. Flask titres were also higher; the net result was tenfold greater production per vessel (Table 3).

Cell numbers being about equal (6.6E7 cells per flask vs. 5E7 per tray) we deduce that per cell expression of sIL6R protein must have been 2–4 times higher in the multilayers. Antigenic specificity and electrophoretic purity of product sIL6R were the same as from control trays.

sIL6R production in serum free medium

Monolayers rapidly rounded up and detached in serum free medium, making tray culture impractical. But detachment in the multilayers was slower, allowing 3 batches be collected (with gentle decanting)

before major areas of bare film were observable with microscope (Table 4). After two months in serum free medium, residual cells were still producing sIL6R. Microscopy *in situ* showed rafts with clumps of rounded cells that were weakly adherent but still viable, as proved by the clumps' rapid ability to re-spread and re-form confluent multilayers inside their carriers when serum was restored to the flask. Selected rafts with clumped colonies could also be picked out of the flask and subcultured with serum to regrow a normal monolayer by methods described previously (Schmitt and Maroudas 1974). Fibronectin did not restore adhesion and spreading of these cells when added to our serum free medium.

sIL6R production in spinner vessel

No problems were encountered with cells shearing off the cellrafts in 2% serum. Three batches were harvested before contamination terminated this test (Table 5). Results appeared to be no better than from static T-flasks (Table 5). For trial production of a few litres of sIL6R, we found the switch to small spinner cumbersome, because we had already made and tested a stock of recycled cellraft in flasks. Thus an extra litre of product simply meant putting five more T150s in the incubator: in line with our usual cytogenetic technique, more flexible in batch size and with less risk of losing the whole litre by contamination.

Surface treatment and recycling

No difference was found between previous dry electrical method and the new wet photochemical method:

Table 2. Production of IL6 receptor in roux bottle

	Volume (ml)	Titre ($\mu\text{g ml}^{-1}$)	Total (batches)	Total yield (mg)
Control tray	75	3.1	4	0.93
Roux + cellrafts	120–250	1.4	15	4.80

Table 3. Production of sIL6R in T-flasks

	Volume (ml)	Titre ($\mu\text{g ml}^{-1}$)	Total (batches)	Total yield (mg per vessel)
Control Tray	75	5.0	3	113
Flask + Cellrafts	200	7.0 ± 1.6	9	1260

Net increase in total yield per vessel = 1,015% Averages from pooled media: 3 pools from 3 batches of 20 trays each, and 10 pools from 9 batches of 6 flasks.

Table 4. Production of IL6R in serum free medium

	Volume (ml)	Titre ($\mu\text{g ml}^{-1}$)	Total (batches)	Total yield (mg per vessel)
Control tray	75	Not feasible	0	0
Serum-free T150s	200	2.8 ± 0.4	3	168

Table 5. Production of IL6 receptor in stirred vessel

	Batch volume (ml)	Titre ($\mu\text{g ml}^{-1}$)	Total (batches)	Total yield (mg per vessel)
500 ml spinner	300	3.3 ± 0.7	3	297

both depended on quality control to confirm that treatment had attained sufficiently low contact angle. But in our laboratory context, the wet method had advantages: it was much slower but treated larger batches, required only standard tissue culture laboratory facilities, and enabled us to recycle our small stock of carriers. Cellrafts were reused for years without deterioration of growth surface, because the hypochlorite dissolved old surface-bound protein as well as proving a good tissue culture treatment for the underlying plastic (with uv, see Fabrication).

Discussion

We have developed a carrier with unusual properties tailored to bulk culture in T-flasks, in order to preserve some of the simplicity and versatility of static incubator technique. We also developed a simple surface treatment/cleaning method for optional recycling of plastic carriers.

Optical quality of the floating multilayers was good enough to make a routine low magnification survey on unstained cells *in situ* (Section Results, Microscopy) and to pick out cellrafts with colonies for subculture (Section Results, sIL6R production in serum free medium). Stained samples at higher power confirmed that cells were fully spread, as on a TC surface (Figure 1d). Brightly fluorescent AO-yellow nuclei indicated where cells were in good condition (Figures 1b and c).

Bulk cell density was low compared to porous carriers (Looby and Griffiths 1988) but might be acceptable for small trial quantities, especially in view of the ease of harvesting seed cells for scaleup. Residual cells after trypsinization regrew to yield multiple cell harvests (Section Results, Cell production). This feature is not found in most porous carriers.

Though initial sIL6R titres were lower (Section Results, Production of sIL6 receptor in roux bottle) the total yield was higher - mainly because the carrier culture was longer lived. This resembles the wellknown longterm viability of cells inside porous carriers, and suggests that immobilized cells can "condition" the local media inside channels at lower densities than previously reported (Kadouri et al.

1992). Titres improved with experience. In the T150 series (Section Results, Production of sIL6R in T-flasks) not only longevity but also titre and protein expression were higher than in monolayer controls, like improvements found with porous carriers (Kadouri et al. 1992).

In serum free medium, productivity was much greater than in monolayer culture; even so, cells tended to round up and wash out though still viable. In this case it might be better to use porous carriers that trap cells even when rounded. On the other hand, tests by Takagi et al. (1999) found that a porous carrier with better spread cells was also more productive. Since cells spread well on multiplanes in serum (Figure 1d) one might try to retain this advantage by seeking more effective adhesion factors for serum free medium (Section Results, sIL6R production in serum free medium).

We conclude that floating multiplanes operate in a niche not occupied by established carrier technology. Having solved our problem of obtaining litre samples from the occasional shear sensitive clone we have no plans to develop cellraft, but samples of the experimental carriers used above are available from corresponding author NGM.

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